University of Alberta

Synchronization of ovulation in cyclic gilts with porcine luteinizing hormone (pLH) and its effects on reproductive function

by

Katherine Lauren Degenstein ©

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Master of Science
in
Animal Science

Department of Agricultural, Food, and Nutritional Science

Edmonton, Alberta
Fall 2007

Library and
Archives Canada

Published Heritage
Branch

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque et
Archives Canada

Direction du
Patrimoine de l'édition

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence
ISBN: 978-0-494-33230-6
Our file Notre référence
ISBN: 978-0-494-33230-6

NOTICE:
The author has granted a non-exclusive license allowing Library and Archives Canada to reproduce, publish, archive, preserve, conserve, communicate to the public by telecommunication or on the Internet, loan, distribute and sell theses worldwide, for commercial or non-commercial purposes, in microform, paper, electronic and/or any other formats.

The author retains copyright ownership and moral rights in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

AVIS:
L'auteur a accordé une licence non exclusive permettant à la Bibliothèque et Archives Canada de reproduire, publier, archiver, sauvegarder, conserver, transmettre au public par télécommunication ou par l'Internet, prêter, distribuer et vendre des thèses partout dans le monde, à des fins commerciales ou autres, sur support microforme, papier, électronique et/ou autres formats.

L'auteur conserve la propriété du droit d'auteur et des droits moraux qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

In compliance with the Canadian Privacy Act some supporting forms may have been removed from this thesis.

While these forms may be included in the document page count, their removal does not represent any loss of content from the thesis.

Conformément à la loi canadienne sur la protection de la vie privée, quelques formulaires secondaires ont été enlevés de cette thèse.

Bien que ces formulaires aient inclus dans la pagination, il n'y aura aucun contenu manquant.

Canada

ABSTRACT

Synchronization of ovulation is an important prerequisite in fixed-time artificial insemination (AI) and embryo transfer technologies. Previous research has shown the reliability of porcine luteinizing hormone (pLH; Lutropin-V, Bioniche) in synchronizing ovulation in weaned sows. This study aimed to evaluate the synchronizing ability of pLH in cyclic gilts, compare pLH to human chorionic gonadotropin (hCG), and assess the effect of hormone treatment on embryo quality. In an initial study, pLH was found to elicit a physiological response, and the average treatment to ovulation interval was 38.4 h after pLH treatment, which tended to be earlier than control animals (46.4 h; $p = 0.06$). A more extensive study confirmed this trend, with pLH and hCG animals ovulating earlier than control animals ($p = 0.001$), with the least variation in time of ovulation among pLH-treated animals. Embryo quality was not affected by hormone treatment. These results indicate that pLH is applicable for use in cyclic gilts, and can reliably synchronize ovulation without detrimentally affecting embryo quality.

ACKNOWLEDGEMENTS

I would like to thank Dr. Michael Dyck for giving me the opportunity to conduct this research, for his ongoing support throughout this project, and for helping me to meet a very tight deadline! It has been a great learning experience and I am grateful for the wonderful opportunity.

Thanks to Dr. George Foxcroft and Dr. Eduardo Beltranena for performing the surgeries and for their advice along the way. As well, I am grateful to Dr. Divakar Ambrose and Dr. Norm Stacey for being part of my committee.

Thank you to Rose O'Donoghue for all her help with preparation and embryo work. Also, thanks to Jenny Patterson, Anke Wellen, Francois Paradis, Alex Pasternak, Sabrina Sarmento, and Miranda Smit for their assistance with animal work and/or embryo collection. I am also grateful for Tamara Staska's work on surgery preparation and assistance. As well, thank you to Shirley Shostak for her work on the assays.

Thank you to the staff at the Swine Research and Technology Centre for their patience and help with animal selection and assistance with this project. To the SRDP and my fellow graduate students, thank you for the advice, numerous suggestions, and support throughout this project. I'd also like to thank Dr. Peter Blenis and Dr. Ellen Macdonald for advice with statistics. A special thanks to Bioniche, NSERC, Alberta Pork, Hypor, ALIDF, and AARI for providing financial support for this project.

Last, but definitely not least, thank you to my family for their encouragement and support. I could not have done it without you!

TABLE OF CONTENTS

GENERAL INTRODUCTION……………………………………………………1
References……………………………………………………………3

CHAPTER 1
LITERATURE REVIEW
1.1 Introduction……………………………………………………6
1.2 Reproductive Technologies…………………………………………11
1.3 Porcine Luteinizing Hormone…………………………………………14
1.4 Hormone Treatment and its Effect on Embryo Quality…………………19
1.5 Morphological Indicators of Embryo Quality……………………………22
1.6 Gene Expression and Embryo Quality…………………………………25
1.7 Conclusions……………………………………………………28
1.8 References……………………………………………………28

CHAPTER 2
ENDOCRINE RESPONSE AND SYNCHRONIZATION OF OVULATION IN CYCLIC GILTS TREATED WITH PORCINE LUTEINIZING HORMONE (pLH)
2.1 Introduction……………………………………………………37
2.2 Materials and Methods……………………………………………38
2.3 Results………………………………………………………43
2.4 Discussion……………………………………………………46
2.5 References……………………………………………………49

CHAPTER 3
THE USE OF PORCINE LUTEINIZING HORMONE (pLH) OR HUMAN CHORIONIC GONADOTROPIN (hCG) IN CYCLIC GILTS TO SYNCHRONIZE OVULATION AND THE EFFECT OF THESE TREATMENTS ON EARLY EMBRYO QUALITY
3.1 Introduction……………………………………………………65
3.2 Materials and Methods……………………………………………67
3.3 Results………………………………………………………72
3.4 Discussion……………………………………………………74
3.5 References……………………………………………………82

CHAPTER 4
GENERAL DISCUSSION AND CONCLUSIONS
4.1 General Discussion………………………………………………103
4.2 Conclusions……………………………………………………109
4.3 References……………………………………………………110

LIST OF TABLES

Table 2.1: Estrus characteristics and timing of ovulation for the peri-estrous periods before and after pLH or saline treatment (Least square means ± SEM)............52

Table 2.2: The relationship between the LH surge and estrus timing, ovulation timing, and pLH or saline treatment (Least square means ± SEM)...........................53

Table 2.3: LH surge characteristics (Least square means ± SEM); LH potencies expressed as ng equivalents of the purified pLH preparation AFP12389A with a stated potency of 2.2 X NIH S1...53

Table 2.4: Gilt reproductive performance at day 6.4 (± 0.3) of gestation (Least square means ± SEM)...53

Table 2.5: Number of embryos, at various stages of development, recovered at day 6.4 (± 0.3) of gestation (Least square means ± SEM)......................................54

Table 3.1: Estrus characteristics and timing of standing estrus and ovulation following pLH, hCG, or saline treatment (Least square means ± SEM)........................87

Table 3.2: Gilt reproductive performance at day 5.9 (± 0.03) of gestation (Least square means ± SEM)...88

Table 3.3: Average number of embryos, by stage of development, recovered at day 5.9 (± 0.03) of gestation (Least square means ± SEM)..................................88

Table 3.4: Embryo characteristics as measured using Metamorph (Least square means ± SEM); number in brackets indicates the number of animals used in the analysis..89

LIST OF FIGURES

Figure 2.1: Outline of the treatment schedule..55

Figure 2.2: Peak plasma LH concentrations during the endogenous or pLH-enhanced LH surge for each gilt; plasma LH concentrations are expressed as ng equivalents of the purified pLH preparation AFP12389A with a stated potency of 2.2 X NIH S1..56

Figure 2.3: Amplitude of the endogenous or pLH-enhanced LH surge for each gilt; plasma LH concentrations are expressed as ng equivalents of the purified pLH preparation AFP12389A with a stated potency of 2.2 X NIH S1.......................56

Figure 2.4: LH profile obtained for the control gilt in litter 1. Arrows indicate the timing of cloprostenol, eCG, and saline treatment and the onset of estrus. The graph is divided into 12-hour (left) and 4-hour (right) blood sampling frequencies. Plasma LH concentrations are expressed as ng equivalents of the purified pLH preparation AFP12389A with a stated potency of 2.2 X NIH S1......................57

Figure 2.5: LH profile obtained for the pLH-treated gilt in litter 1. Arrows indicate the timing of cloprostenol, eCG, and pLH treatment and the onset of estrus. The graph is divided into 12-hour (left) and 4-hour (right) blood sampling frequencies. Plasma LH concentrations are expressed as ng equivalents of the purified pLH preparation AFP12389A with a stated potency of 2.2 X NIH S1..................57

Figure 2.6: LH profile obtained for the control gilt in litter 2. Arrows indicate the timing of cloprostenol, eCG, and saline treatment and the onset of estrus. The graph is divided into 12-hour (left) and 4-hour (right) blood sampling frequencies. Plasma LH concentrations are expressed as ng equivalents of the purified pLH preparation AFP12389A with a stated potency of 2.2 X NIH S1......................58

Figure 2.7: LH profile obtained for the pLH-treated gilt in litter 2. Arrows indicate the timing of cloprostenol, eCG, and pLH treatment and the onset of estrus. The graph is divided into 12-hour (left) and 4-hour (right) blood sampling frequencies. Plasma LH concentrations are expressed as ng equivalents of the purified pLH preparation AFP12389A with a stated potency of 2.2 X NIH S1...................58

Figure 2.8: LH profile obtained for the control gilt in litter 3. Arrows indicate the timing of cloprostenol, eCG, and saline treatment and the onset of estrus. The graph is divided into 12-hour (left) and 4-hour (right) blood sampling frequencies. Plasma LH concentrations are expressed as ng equivalents of the purified pLH preparation AFP12389A with a stated potency of 2.2 X NIH S1.....................59

Figure 2.9: LH profile obtained for the pLH-treated gilt in litter 3. Arrows indicate the timing of cloprostenol, eCG, and pLH treatment and the onset of estrus. The graph is divided into 12-hour (left) and 4-hour (right) blood sampling frequencies.

Plasma LH concentrations are expressed as ng equivalents of the purified pLH preparation AFP12389A with a stated potency of 2.2 X NIH S1....................59

Figure 2.10: LH profile obtained for the control gilt in litter 4. Arrows indicate the timing of cloprostenol, eCG, and saline treatment and the onset of estrus. The graph is divided into 12-hour (left) and 4-hour (right) blood sampling frequencies. Plasma LH concentrations are expressed as ng equivalents of the purified pLH preparation AFP12389A with a stated potency of 2.2 X NIH S1....................60

Figure 2.11: LH profile obtained for the pLH-treated gilt in litter 4. Arrows indicate the timing of cloprostenol, eCG, and pLH treatment and the onset of estrus. The graph is divided into 12-hour (left) and 4-hour (right) blood sampling frequencies. Plasma LH concentrations are expressed as ng equivalents of the purified pLH preparation AFP12389A with a stated potency of 2.2 X NIH S1....................60

Figure 2.12: LH profile obtained for the control gilt in litter 3 immediately following saline treatment (at 4:00 PM). Samples were taken at 30 minute intervals for four hours. Plasma LH concentrations are expressed as ng equivalents of the purified pLH preparation AFP12389A with a stated potency of 2.2 X NIH S1..............61

Figure 2.13: LH profile obtained for the pLH-treated gilt in litter 3 immediately following pLH treatment (at 4:00 PM). Samples were taken at 30 minute intervals for four hours. Plasma LH concentrations are expressed as ng equivalents of the purified pLH preparation AFP12389A with a stated potency of 2.2 X NIH S1....61

Figure 2.14: LH profile obtained for the control gilt in litter 4 immediately following saline treatment (at 4:00 PM). Samples were taken at 30 minute intervals for four hours. Plasma LH concentrations are expressed as ng equivalents of the purified pLH preparation AFP12389A with a stated potency of 2.2 X NIH S1..............62

Figure 2.15: LH profile obtained for the pLH-treated gilt in litter 4 immediately following pLH treatment (at 4:00 PM). Samples were taken at 30 minute intervals for four hours. Plasma LH concentrations are expressed as ng equivalents of the purified pLH preparation AFP12389A with a stated potency of 2.2 X NIH S1....62

Figure 2.16: Average numbers of embryos recovered at each stage of development for each treatment at day 6.4 (± 0.3) of gestation. It is important to note that one control sow was euthanized one day early (day 4.8)..................................63

Figure 2.17: An example of a differentially stained expanded blastocyst. Cells appearing pink are stained with propidium iodide, cells appearing blue are stained with bisbenzimide, and cells appearing purple are stained with both...............64

Figure 3.1: Outline of the treatment schedule..89

Figure 3.2: Variation in timing of ovulation following saline, hCG, or pLH treatment (Figure generated in SAS and edited for format). ^{a,b} values with different superscripts are significantly different (p = 0.001); ^{c,d} values with different superscripts are significantly different (p = 0.03); ^{e,f} values with different superscripts are different (p = 0.06)..90

Figure 3.3: Plasma progesterone concentrations for each animal within a littermate set at day 5.9 (± 0.03) of gestation..91

Figure 3.4: Relationship between ovulation rate and plasma progesterone concentration at day 5.9 (± 0.03) of gestation (n = 30)..92

Figure 3.5: Relationship between ovulation rate and plasma progesterone concentration at day 5.9 (± 0.03) of gestation separated by treatment (n = 30)....................93

Figure 3.6: Representative pictures of early (A, B) and non-expanded blastocysts (C, D)..94

Figure 3.7: Representative pictures of expanded blastocysts...............................95

Figure 3.8: Representative pictures of de-expanded (A, B) and hatched blastocysts (C, D)..96

Figure 3.9: Examples of differentially stained expanded blastocysts. Cells appearing pink are stained with propidium iodide, cells appearing blue are stained with bisbenzimide, and cells appearing purple are stained with both.....................97

Figure 3.10: Relationship between individual expanded blastocyst diameter and total cell number 98

Figure 3.11: Relationship between individual expanded blastocyst diameter and total cell number separated by treatment..99

Figure 3.12: The stage of the embryos recovered for each treatment at day 5.9 (± 0.03) of gestation..100

Figure 3.13: Relationship between the number of expanded and hatched blastocysts recovered at day 5.9 (± 0.03) of gestation, and ovulation rate 101

Figure 3.14: Relationship between the number of expanded and hatched blastocysts recovered at day 5.9 (± 0.03) of gestation, and the rate of recovery 102

LIST OF ABBREVIATIONS

AI	Artificial insemination
ART	Assisted reproductive technologies
BSA	Bovine serum albumin
BTS	Beltsville thawing solution
BW	Body weight
CD44	Receptor for hyaluronic acid
CIDR	Controlled internal drug release device
CL	Corpus luteum
DAPI	4',6-Diamidino-2-phenylindole dihydrochloride
eCG	Equine chorionic gonadotropin; previously known as PMSG
FSH	Follicle-stimulating hormone; pFSH is the porcine form
GAG	Glycosaminoglycan
GnRH	Gonadotropin-releasing hormone
HA	Hyaluronic acid
hCG	Human chorionic gonadotropin
HIP	Receptor for heparin
ICM	Inner cell mass
IETS	International Embryo Transfer Society
IGF-I	Insulin-like growth factor I
IGF-IR	Insulin-like growth factor I receptor
IM	Intramuscularly
IU	International units
IVF	*In vitro* fertilization
IVM	*In vitro* maturation
LH	Luteinizing hormone; pLH is the porcine form
LHRH	Luteinizing hormone releasing hormone; also known as GnRH
ME	Metabolic energy
mRNA	Messenger ribonucleic acid
NRC	National Research Council

PBS	Phosphate buffered saline
PG600	Combination of 400 IU eCG and 200 IU hCG
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PMSG	Pregnant mares' serum gonadotropin; now known as eCG
PVP	Polyvinylpyrrolidone
RIA	Radioimmunoassay
RT-PCR	Reverse transcriptase polymerase chain reaction
SAS	Statistical Analysis System
SEM	Standard error of the mean
SRTC	Swine Research and Technology Centre
TE	Trophectoderm
TUNEL	Terminal deoxynucleotidyl transferase mediated dUDP nick-end labeling
WEI	Weaning to estrus interval

GENERAL INTRODUCTION

Control of follicular development and ovulation has practical implications for several aspects of livestock management and for many reproductive technologies. Variation among females in the duration of standing estrus, which has direct implications for timing of ovulation, has been well established in swine (Soede and Kemp 1997). Although a relationship between the time of ovulation after the onset of estrus and estrus duration has been observed in both sows (Nissen *et al.* 1997) and gilts (Almeida *et al.* 2000), little else (onset of estrus, duration of 'man-estrus', vulval reddening) seems to relate to the timing of ovulation (Langendijk *et al.* 2000). Even though this relationship exists, Soede *et al.* (1995) illustrated that there is substantial variation among animals as to when ovulation occurs during estrus (range of 39 to 133% in sows for timing of ovulation in relation to total duration of estrus). The recorded onset and duration of estrus also depends on the frequency of estrus detection (Almeida *et al.* 2000). Therefore, the appropriate timing of insemination relative to ovulation can be problematic, particularly when this parameter can have a large influence on subsequent fertility (Soede *et al.* 1995). Numerous aspects of production can be affected, ranging from routine breeding in a production setting, to the use of fixed-time artificial insemination (AI), which is specific timing of an insemination following synchronization of ovulation.

Although it has been clear for some time that the timing of ovulation is an important issue for the swine industry and extensive research has been ongoing, a standardized method of controlling ovulation has not been established. Chapter 1 of this thesis reviews approaches that have been taken to address this problem, and introduces new concepts and products that have emerged recently. These approaches will also be discussed in relation to developing technologies that depend greatly on the control of ovulation.

Much of the difficulty in controlling ovulation in pigs is due to the fact that they are a litter-bearing species and, therefore, numerous oocytes are ovulated simultaneously. Consequently, unlike cattle or sheep, ovulation is not a discreet event and occurs over an extended period of time of 1 to 4 hours (Soede *et al.* 1992). The duration of multiple ovulations can have implications for proper insemination timing. It has also been suggested that an extended ovulation interval can influence the make-up of the litter in regards to the range of embryonic development obtained (Pope *et al.* 1988), although this suggestion has been disputed (Soede *et al.* 1992, 1993). Embryonic stage heterogeneity, as suggested by Pope *et al.* (1986), can affect embryonic survival. On the other hand, it may have implications for uterine crowding (Geisert and Schmitt 2002). A recent study by Vinsky *et al.* (2007) defined a population of animals that have this type of developmental variability regardless of any experimental treatment. If diversity in embryonic stage of development is in fact detrimental to embryo survival, it would be of interest to control this. This may be possible through use of exogenous hormone treatment if given at the appropriate time (Lambert *et al.* 1991). For embryo transfer, embryonic heterogeneity is undesirable, as it reduces the number of embryos at the appropriate developmental stage for transfer (Hazeleger and Kemp 1994). Therefore, the use of exogenous hormone treatment is a necessary component of embryo transfer procedures.

When controlling ovulation in livestock, pharmacological approaches are often used (Estill 1999). In swine, the most established treatment protocol involves a combination of equine chorionic gonadotropin (eCG) to stimulate follicular growth followed by human chorionic gonadotropin (hCG) to trigger ovulation (Webel and Day 1982). A review of the literature revealed many variations on this procedure (Estill 1999), suggesting that no single approach has been finalized in which consistent results are delivered. With this in mind, it is still uncertain if it is possible to reliably control ovulation in pigs. The research presented here aims to evaluate if porcine luteinizing hormone (pLH; Lutropin-V, Bioniche Animal Health Canada Inc., Belleville, Ontario, Canada) is an appropriate product for inducing consistent and reliable control of ovulation in swine. Promising results have been obtained using pLH in weaned sows (Candini *et al.* 2001; Viana *et al.* 2002; Cassar *et al.* 2005), but to date research involving this product is limited, particularly with regard to its application in pubertal and cyclic gilts.

The studies described here concentrated on evaluating the application of pLH for ovulation induction in post-pubertal, cyclic gilts. Initially, the response of gilts to pLH treatment was evaluated to fully understand the physiological basis of this product's ability to influence ovulation timing. This research is described in Chapter 2 of this thesis. Given that hCG has been the hormone most commonly used to synchronize porcine ovulation, the research described in Chapter 3 compares the synchronizing ability of pLH and hCG, and examines whether pLH offers any specific benefits. The work described in Chapter 3 also addresses the effect of hormonal stimulation of ovulation on embryonic heterogeneity and quality. Hormonal control of ovarian activity in swine has been associated with compromised embryo quality, viability (Ziecik *et al.* 2005; do Lago *et al.* 2005) and cell number (Hazeleger *et al.* 2000). Therefore, embryonic expansion and cell number were evaluated here as indicators of quality to determine if the viability of embryos produced was affected following hormonal ovulation induction. Overall, this thesis aimed to establish if consistent and reliable control of ovulation can be achieved in swine and examined the consequences of hormonal treatment on the resulting embryos.

References

Almeida, F.R.C.L., Novak, S., Foxcroft, G.R. 2000. The time of ovulation in relation to estrus duration in gilts. *Theriogenology.* 53:1389-1396.

Candini, P.H., Moretti, A.S., Silveria P.R.S., Zanella, E., Viana, C.H.C., Santos, I.C.C. 2001. Utilization of a protocol of synchronization of ovulation, induced by porcine LH, to perform insemination programs in fixed time, with one or two inseminations per sow. Sixth International Conference on Pig Reproduction, Columbia MO. pp. 99.

Cassar, G., Kirkwood, R.N., Bennett-Steward, K., Friendship, R.M. 2005. Towards timed single insemination in sows. Proceedings of the 36th Annual Meeting of the American Association of Swine Veterinarians, Toronto, ON. pp. 353-356.

do Lago, V., Vianna, W.L., Gama, R.D., de Campos Rosseto, A., Pinese, M.E., de Sant'Anna Moretti, A. 2005. Second oestrus synchronization and precocious embryo viability after puberty induction in gilts by the use of gonadotrophin treatment. *Reprod. Dom. Anim.* 40:141–144.

Estill, C.T. 1999. Current concepts in estrus synchronization in swine. Proceedings of the American Society of Animal Science. Southern Section ASA Meeting, Memphis, TN. E38.

Geisert, R.D., Schmitt, R.A.M. 2002. Early embryonic survival in the pig: Can it be improved? *J. Anim. Sci.* (E. Supplement 1) 80:E54-E65.

Hazeleger, W., Bouwman, E.G., Noordhuizen, J.P.T.M., Kemp, B. 2000. Effect of superovulation induction on embryonic development on day 5 and subsequent development and survival after nonsurgical embryo transfer in pigs. *Theriogenology.* 53:1063-1070.

Hazeleger, W., Kemp, B. 1994. Farrowing rate and litter size after transcervical embryo transfer in sows. *Reprod. Dom. Anim.* 29:481-487.

Lambert, E., Williams, D.H., Lynch, P.B., Hanrahan, T.J., McGeady, T.A., Austin, F.H., Boland, M.P., Roche, J.F. 1991. The effects of administration of gonadotrophins and estrogens on prenatal survival in gilts. *Theriogenology.* 36:77-85.

Langendijk, P., Soede, N.M., Bouwman, E.G., Kemp, B. 2000. Responsiveness to boar stimuli and change in vulvar reddening in relation to ovulation in weaned sows. *J. Anim. Sci.* 78:3019-3026.

Nissen, A.K., Soede, N.M., Hyttel, P., Schmidt, M., D'Hoore, L. 1997. The influence of time of insemination relative to time of ovulation on farrowing frequency and litter size in sows, as investigated by ultrasonography. *Theriogenology.* 47:1571-1582.

Pope, W.F., Lawyer, M.S., Nara, B.S., First, N.L. 1986. Effect of asynchronous superinduction on embryo survival and range of blastocyst development in swine. *Biol. Reprod.* 35:133-137.

Soede, N.M., Kemp, B. 1993. In synchronized pigs, the duration of ovulation is not affected by insemination and is not a determinant for early embryonic diversity. *Theriogenology.* 39:1043-1053.

Soede, N.M., Kemp, B. 1997. Expression of oestrus and timing of ovulation in pigs. *J. Reprod. Fertil. Suppl.* 52:91-103.

Soede, N. M., Noordhuizen, J.P.T.M., Kemp, B. 1992. The duration of ovulation in pigs, studied by transrectal ultrasonography, is not related to early embryonic diversity. *Theriogenology.* 38:653-666.

Soede, N.M., Wetzel, C.C.H., Zondag, W., de Koning, M.A.I., Kemp, B. 1995. Effects of time of insemination relative to ovulation, as determined by ultrasonography, on fertilization rate and accessory sperm count in sows. *J. Reprod. Fertil.* 104:99-106.

Viana, C.H.C., Candini, P.H., Gama, R.D., Carbone, A., Santos, I.C.C., Vianna, W.L., Barnabe, R.C. 2002. Evaluation of a synchronization of ovulation protocol in cyclic sows. Proceedings of the International Pig Veterinary Congress, Ames, Iowa #678.

Vinsky, M.D., Paradis, F., Dixon, W.T., Dyck, M.K., Foxcroft, G.R. 2007. Ontogeny of metabolic effects on embryonic development in lactating and weaned primiparous sows. *Reprod. Fertil. Dev.* 19:603-611.

Webel, S.K., Day, B.N. 1982. The control of ovulation. In Control of Pig Reproduction. Cole, D.J.A, Foxcroft, G.R. Eds. pp. 197-210.

Ziecik, A.J., Biallowicz, M., Kaczmarek, M., Demianowicz, W., Rioperez, J., Wasielak, M., Bogacki, M. 2005. Influence of estrus synchronization of prepubertal gilts on embryo quality. *J. Reprod. Dev.* 51:379-384.

CHAPTER 1
LITERATURE REVIEW

1.1 Introduction

Estrus synchronization is one of the most important tools producers can use to effectively introduce new animals or reintroduce weaned animals into a breeding herd to meet breeding targets, thus reducing the need for a larger inventory of replacement animals. This, and more specifically synchronization of ovulation, is an essential part of developing reproductive technologies in pig production, such as fixed-time artificial insemination (AI) protocols and embryo transfer. As a result, methods of improving estrus timing and ovulation synchronization will ultimately benefit many areas of swine management.

Follicular development and ovulation are controlled by follicle stimulating hormone (FSH) and luteinizing hormone (LH), respectively, which are released from the pituitary gland under the control of the hypothalamus (Foxcroft and Van de Wiel 1982). The pattern and timing of FSH and LH release will establish when the animal exhibits estrus and ovulates. The main central nervous system control of these hormones is through gonadotropin-releasing hormone (GnRH), which is released by the hypothalamus (Ellendorff and Parvizi 1982). A negative feedback loop involving steroid release from the ovary modulates the pattern of gonadotropin release throughout the estrous cycle, whereas prolonged exposure to estrogen during the follicular phase induces positive feedback to initiate the LH surge that triggers ovulation (Elsasser 1982). Animals do not establish this cyclic pattern of release of the reproductive hormones until they attain puberty when the hypothalamic-pituitary-ovarian axis reaches maturity. In the domestic pig, the female generally reaches puberty at approximately 6 to 7 months of age (approximately 180 to 210 days), and there can be substantial variation in this timing among animals (Tummaruk *et al.* 2007). For example, when evaluating effects of boar exposure on puberty attainment starting at either 135 or 160 days of age, the range at which puberty was reached was 132 to 210 days of age (Patterson *et al.* 2002a; Patterson *et al.* 2002b). Sexual maturity can also be influenced by the interaction of nutrition, genetics, social environment, and boar exposure (Hughes 1982).

As mentioned previously, ovulation is initiated by an LH surge in response to high estrogen secretion from the ovary (Elsasser 1982). As reviewed by Foxcroft and Van de Wiel (1982), it appears that the pre-ovulatory LH surge of approximately 6 ng/ml (based on the assay described in Van de Wiel *et al.* 1981) occurs 40 to 48 hours prior to ovulation and lasts for approximately 20 hours. Peak surge concentrations of LH are relatively low compared to other domestic farm species, but the duration of the LH surge is significantly longer (Foxcroft and Van de Wiel 1982). The onset of estrus can occur anywhere between 8 to 12 hours before to 12 hours after the beginning of the LH surge (Tilton *et al.* 1982; Foxcroft and Van de Wiel 1982), leading to substantial variation in the timing of ovulation relative to the onset of estrus. Clearly, with such variation in the relationship between estrus and ovulation, it would be very valuable to accurately establish the timing of ovulation regardless of the onset of standing estrus.

There have been numerous attempts to control and alter hormone release in the cycling female. Early efforts to control the release of these hormones in pigs involved manipulating lactation length. A phenomenon observed in swine, known as lactational anestrus, is characterized as a quiescence of the ovaries during lactation, which is thought to be due to lack of an episodic pattern of LH secretion (Foxcroft *et al.* 1995). Upon weaning, episodic LH secretion is re-established, resuming the estrous cycle in a predictable fashion. However, very short lactations (8 to 12 days) tend to increase the weaning to estrus interval (WEI) and result in lower fertility, decreasing the benefit that would be expected with a short lactation (Estill 1999).

Progestagens have been used to mimic plasma progesterone concentrations seen in the luteal phase of the estrous cycle and during pregnancy. Progesterone is naturally released by the corpus luteum (CL) at approximately day 3 to 4 of the cycle, with day 0 being the first day of standing estrus, and then later declines between day 10 and 15 (Foxcroft and Van de Wiel 1982). Exogenous progestagens attempt to synchronize estrus of already cyclic animals by artificially extending the luteal phase and thus inhibiting follicular growth. Upon withdrawal of the progestagen, follicular growth is reinitiated and the reproductive cycle progresses in a predictable manner (Estill 1999). Progesterone implants have also been used for this purpose and upon removal resulted in a prompt return to estrus (Estill 1999). Treatment with altrenogest, an orally active progestin, mimics the luteal phase of the estrous cycle and maintains a population of small and medium follicles during treatment (Guthrie and Bolt 1985). Feeding altrenogest to either weaned sows or gilts at an unknown stage of the estrous cycle for 14 days, results in a larger number of animals returning to estrus within 7 to 10 days of withdrawal when compared to control animals, with no negative effects on fertility (Wood *et al.* 1992). In gilts, the recommended dosage of this product is 15 to 20 mg for 14 to 18 days, and it is only successful in synchronizing already cyclic animals (Webel and Day 1982).

Prostaglandins have also been applied to synchronization protocols by inducing coordinated luteolysis of the CL and re-initiating the cycle at the same point in a group of animals (Estill 1999). Because the luteal phase dictates the length of the estrous cycle (Foxcroft and Van de Wiel 1982), it is the most logical phase for interventions which significantly alter the overall length of the cycle. However, the degree to which the luteal phase can be shortened is limited in swine, as the porcine CL has a reduced sensitivity to prostaglandins for the first 12 days of the luteal phase (Ziecik 2002), and naturally starts regressing at day 15 (Bazer *et al.* 1982). If exogenous prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is given in this window of time, luteolysis can be achieved. Two treatments of $PGF_{2\alpha}$ 12 hours apart provide better results when compared to a single treatment (Pressing *et al.* 1987). On the other hand, Estill *et al.* (1993) was able to induce luteolysis at approximately day 10 with repeated treatments of $PGF_{2\alpha}$ staring at day 5, resulting in a return to estrus around day 13. Therefore, the CL may be responsive to $PGF_{2\alpha}$, but only after repeated exposure. $PGF_{2\alpha}$ has also been used to eliminate the CL in early pregnancy, resulting in the loss of pregnancy and a predictable return to estrus (Webel and Day 1982). However, such protocols resulting in abortion are controversial.

More recently, treatments with exogenous hormones have been applied to mimic the natural release of FSH and/or LH, resulting again in predictable estrus timing. These protocols generally use a combination of equine chorionic gonadotropin hormone (eCG; also known as pregnant mares' serum gonadotrophin- PMSG) and human chorionic gonadotropin (hCG), which act similarly to FSH and LH, respectively. Hormone treatments have also been used in combination with other synchronization techniques, such as batch weaning and prostaglandin treatment (Estill 1999). Typically, eCG is given at an appropriate time to stimulate follicular growth (i.e. after weaning) and is then followed by hCG 72 to 96 hours after to stimulate ovulation (Webel and Day 1982). A study by Hunter (1967) found that treatment with hCG resulted in ovulation around 42 hours following treatment. Treatment with hCG was found to stimulate a longer duration of ovulation than what was observed in non-stimulated animals (Soede *et al.* 1992). Also, Pope *et al.* (1988) observed that hCG-treated sows began to ovulate at 39 to 41 hours following treatment, ovulating the majority of their follicles over a short period of time; however, the overall duration of ovulation was not affected. This extended interval could result in more embryo heterogeneity, with embryos being at many different stages of development. This was illustrated by Xie *et al.* (1990) who found that oocytes from later ovulating follicles became the less developed embryos of the litter. Therefore, given these varying results, more study is needed to determine if indeed embryonic uniformity can be influenced by ovulation induction.

GnRH has also been used to manipulate ovulation. In a recent study, the onset of ovulation occurred at 29 to 38.5 hours after treatment with a GnRH analogue (D-Phe^6-LHRH), with an average timing of 36.4 hours (Brussow *et al.* 1996). Pregnancy rate and the number of piglets born were also improved with the use of the same GnRH agonist when compared to hCG (Brussow *et al.* 1996). Other studies confirmed greater precision when using GnRH or GnRH analogues versus hCG to induce ovulation (see the review of Estill 1999), most likely due to GnRH's action directly on the pituitary gland, immediately stimulating LH release in a predictable fashion (Brussow *et al.* 1996).

A gel product, containing the GnRH agonist Triptorelin, has been developed and is administered intravaginally (Roski 2004). In theory, the slow release and absorption of the GnRH agonist from the gel vehicle should improve its effectiveness in inducing ovulation and avoid the need for multiple GnRH treatments to mimic natural GnRH release. A trial with this product in sows by Roski (2004) found that the variation in mean ovulation duration was decreased with higher viscosity gel when compared to control animals. However, there was no difference in reproductive parameters or LH characteristics among treatments (Roski 2004). Another study involving this GnRH product decreased the onset of estrus in weaned sows if it was given at a fixed time after weaning, but there was no advantage if the product was given after the onset of estrus (Knox *et al.* 2003). This study also found that the timing of insemination for animals receiving treatment was less than optimal in relation to timing of ovulation. Therefore, treatment or insemination timing may need to be re-evaluated (Knox *et al.* 2003). GnRH treatment was again shown to improve the number of sows expressing estrus and ovulating within six days of weaning, and when using a fixed-time insemination, high pregnancy rates were achieved (Baer and Bilkei 2004). However, three inseminations were used in this study, which would typically not be the case in a fixed-time insemination protocol. In any case, it appears that the addition of either GnRH or hCG helps induce a more predictable timing of ovulation, while products such as PG600 (a combination of eCG and hCG, Intervet Canada Inc., Whitby, Ontario, Canada) reliably induce estrus but not necessarily consistent ovulation patterns (De Rensis *et al.* 2003).

The need for the inclusion of LH or GnRH-like agents in stimulation protocols was summarized by Guthrie (2005), and is based on the information that porcine follicles become dependent on LH at approximately day 3 of the follicular phase. This is due to a decrease in FSH receptor mRNA expression in granulosa cells and a coincident and dramatic increase of LH receptor mRNA expression in both granulosa and theca cells at this point; by day 5, FSH receptor mRNA expression is no longer present (Guthrie 2005). It has been suggested that this LH dependence can occur earlier, possibly in follicles that are greater than 2 mm in size (Guthrie 2005). LH also stimulates the oocyte to complete nuclear maturation by resuming/completing meiosis (Hunter 2000). Therefore, for stimulation protocols to be successful in promoting follicular growth and ovulation, a LH component is clearly important.

1.2 Reproductive Technologies

Assisted reproductive technologies (ART's) have recently come into more mainstream use, and with regards to swine, this is mostly due to the prevalent use of artificial insemination (AI). This approach is highly dependent on detection of estrus to reliably time the insemination and, therefore, requires a significant investment in labour. Yet, with the use of exogenous hormones, the time of ovulation can be controlled and appropriately timed inseminations can be carried out without substantial estrus detection, although boar exposure is still recommended (Langendijk *et al.* 2005). This has led to the development of fixed-time AI protocols. An extension of exogenous hormone use and fixed-time AI protocols is its' role in successful embryo transfer procedures.

In cattle, well-developed fixed-time AI protocols have been established and are commonly applied. These protocols are of interest, as synchronization can allow for shortening of the calving season and reduce labour requirements. As well, these protocols facilitate optimally timed inseminations when it is typically difficult to detect estrus (Larson *et al.* 2006). As discussed in a review by Bo *et al.* (2002), prostaglandins ($PGF_{2\alpha}$) have been used most commonly to synchronize estrus in cattle; however, the ability of $PGF_{2\alpha}$ treatment to influence the emergence of a new follicular wave depends on the stage of the dominant follicle at the time prostaglandin is administered. Therefore, gonadotropin hormone treatments (i.e. GnRH in the Ovsynch treatment) in combination with $PGF_{2\alpha}$ have been used to first trigger the emergence of a new follicular wave and possibly eliminate the dominant follicle (if present) through induced ovulation, then the subsequent CL using $PGF_{2\alpha}$, followed by induced ovulation with further gonadotropin treatment. To avoid unplanned/early ovulation, a progestagen treatment has also been included. An experiment by Larson *et al.* (2006) compared pregnancy rates between fixed-time inseminations, versus inseminating when standing estrus was observed, with a protocol similar to the Ovsynch treatment being used in both situations. First, it was found that the inclusion of a progestagen improved pregnancy rates (from 43% to 54%) in the protocol that did not require estrus detection. It also improved estrus detection for the protocol that required it for insemination (increase of 9%). Secondly, using timed AI without estrus detection was as efficient as checking for estrus, breeding upon standing estrus, and then using timed AI to 'clean-up' those who did not show estrus, thus effectively removing problems with estrus detection in these animals. The success of these protocols in cattle demonstrates the advantages that they can bring to livestock breeding management.

Pharmacological intervention in swine is more limited compared to cattle; however, in principle, it could be used quite successfully for prepubertal and pubertal gilts and weaned sows (Estill 1999). Fixed-time AI has also been used in swine to reduce labour requirements for estrus detection (Baer and Bilkei 2004). A fixed-time protocol using GnRH agonist treatment utilizing the gel vehicle 96 hours after weaning resulted in more animals expressing estrus and ovulating within 6 days of weaning, as well as better pregnancy rates and larger litters (Baer and Bilkei 2004). Another fixed-time AI study found intramuscular GnRH treatment reduced WEI, but timing of the insemination was incorrect, resulting in lower farrowing rates (Pereira *et al.* 2001). It is clear that the timing of insemination following hormonal synchronization must be carefully considered. For pigs at an unknown point in their estrous cycle, prostaglandins cannot be used to the same extent as in cattle. As mentioned previously, the porcine CL is insensitive to its action for the first 12 days of the luteal phase (Ziecik 2002), restricting the extent to which the cycle can be shortened. As a result, for pigs, it is essential to know the stage of an animals' estrous cycle in order to efficiently employ these protocols.

As with fixed-time AI, embryo transfer has been applied quite successfully in cattle. The main focus of these protocols is to 'reproduce and improve desirable genetics' (Bo *et al.* 2002), as well as to address biosecurity concerns. Ovsynch and similar protocols improved pregnancy rates in recipient embryo transfer animals and eliminated the need for estrus detection. To increase the chances of pregnancy in bovine embryo transfer, FSH or eCG have been used to superovulate recipient animals, resulting in multiple CL's (Bo *et al.* 2002). Although the primary objective of superovulation is to obtain several embryos from the donor animal, one benefit of superovulating recipient animals is producing multiple CL's, resulting in higher concentrations of progesterone which enhances pregnancy establishment (Bo *et al.* 2002). Studies have indeed confirmed this hypothesis, with eCG treatment increasing progesterone concentrations and pregnancy rates (Bo *et al.* 2002). Other studies, on the other hand, indicate that eCG treatment, due to its long half life, can result in an increase of large unovulated follicles (Walsh *et al.* 1993) which may impact early embryonic development by creating a hostile uterine environment (Boland *et al.* 1978). When the hormone pFSH was used in similar protocols in place of eCG, and it was shown to induce superovulation and produce large numbers of transferable embryos when given in multiple injections (Staigmiller *et al.* 1995), while producing fewer large unovulated follicles (Goulding *et al.* 1991). It also was observed that an increasing frequency of pFSH injections improved the superovulatory response (Walsh *et al.* 1993). This is most likely due to the short half life of pFSH in cattle (5 hours) which results in the need for multiple injections to maintain a high level of FSH (Demoustier *et al.* 1988). This multiple injection protocol is not ideal, and may be improved upon with the development of a carrier polyvinylpyrrolidone (PVP) which would reduce the number of injections required as it has been observed in ewes (D'Alessandro *et al.* 2001). It must be noted that high degrees of individual variability in response to either eCG or pFSH have been observed; however, when controlling these differences by performing the experiment during one season, using a large sample size, the same breed, and animals of similar age, the differences between eCG and pFSH persist (Goulding *et al.* 1991).

Embryo transfer protocols in swine also typically use a combination of eCG and hCG. For this technique, exogenous hormone treatment is not only used to synchronize ovulation, but has also been used to induce superovulation, facilitating the collection of large numbers of embryos (Hazeleger *et al.* 1994a). In this case, 2000 IU of eCG was considered a superovulating dose, followed 72 hours later by 750 IU of hCG. Superovulation occurs because of the ability of the exogenous hormones to rescue follicles that would normally undergo atresia (Liu *et al.* 2003). Conversely, reasonable numbers of embryos can be obtained and successful transfers into synchronized embryo recipients can be achieved without the need for such a high dose of eCG, as seen in an experiment by Hazeleger and Kemp (1994b) in which they used 1000 IU eCG and 750 IU hCG 72 hours later. The factor that seemed to influence whether a pregnancy was established in this case was the stage of the embryo (i.e. blastocyst versus less developed). Typically, embryos move from the oviduct to the uterine horn at the four cell stage, at approximately 60 to 72 hours after the onset of estrus (Bazer *et al.* 1982). By day 5, the embryos should be at the blastocyst stage and begin hatching at days 6 to 7 (Bazer *et al.* 1982). Even though all animals were treated the same in the Hazeleger and Kemp study (1994b), a wide range of embryonic developmental stages was obtained, which may be due to a longer duration of ovulation stimulated by hCG. Therefore, an alternate treatment that shortens the duration of ovulation would be expected to result in more embryos at a single stage (preferably blastocysts). When selecting blastocysts, Hazeleger *et al.* (2000) recommended that embryos with larger diameters (158 µm or larger) be used, as they found that those embryos give higher pregnancy rates. They suggest that diameter is an indicator of blastocoel formation which may reflect embryo functioning.

1.3 Porcine Luteinizing Hormone

Recently, a new porcine LH (pLH) product has become available from Bioniche Animal Health Canada Inc. (Belleville, Ontario, Canada) under the trade name Lutropin-V. This is a highly purified hormone preparation, isolated from the porcine pituitary gland, with little cross contamination with pFSH (Lutropin-V product insert). Since pLH is a natural porcine hormone, it is expected to stimulate ovulation more reliably in swine compared to hCG. As previously stated, it is a LH surge that initiates ovulation, and with exogenous pLH treatment, the threshold level of LH could conceivably be reached more quickly, resulting in a more coordinated ovulation over a shorter period of time. Natural LH clearance is very rapid, with one study recording a 30.9 minute half-life for LH in gilts immunized against GnRH (Esbenshade *et al.* 1986). However, injected pLH given at an appropriate time may be able to work in conjunction with natural LH release, allowing the LH surge to be initiated or increased more rapidly, after which it would be quickly eliminated from the system. hCG has a half-life of approximately 30 hours in gilts (Stone *et al.* 1987) due to an additional C-terminal peptide (Garcia-Campayo and Boime 2001) and its high affinity binding to the LH receptor (Dufau 1998). As a result, it may continue to stimulate ovulation of follicles over a longer period of time before being cleared from circulation.

In cattle, pLH has been used in fixed-time AI protocols as a replacement for hCG or GnRH to induce ovulation following progestagen treatment and removal. Ovulation occurred between 24 and 36 hours after pLH treatment and high numbers of embryos were obtained following fixed-time AI (Baruselli *et al.* 2006). One study using pLH in an Ovsynch-like protocol found that pLH treatment raised LH concentrations for a longer period of time compared to the induced response to GnRH, before returning to basal levels (Ambrose *et al.* 2005). Also, early in the cycle, treatment with high doses of pLH resulted in higher progesterone concentrations (Ambrose *et al.* 2005). This increase in progesterone may be beneficial for maintenance of pregnancy, especially in cattle which typically possess only one CL. This is supported by a study by Lopez-Gatius *et al.* (2004) who found that progesterone supplementation improved maintenance of pregnancy. However, it is clear in a study conducted by Martinez *et al.* (1999) that the ability of pLH or GnRH administration to induce ovulation depends heavily on the stage of the dominant follicle, and the initial response to ovulation induction will have implications for the entire synchronization protocol. Use of pLH in combination with $PGF_{2\alpha}$ has not been seen to be beneficial in cattle with regard to increasing ovulation rate for embryo transfer, but it is useful in synchronizing ovulation (Bo *et al.* 2006). Depending on the breed, pLH administration also may need to be delayed, as it appears that rates of follicular development are variable among breeds and if the pLH treatment is too early, lesser quality embryos result (Bo *et al.* 2006).

In swine, an initial experiment using pLH to synchronize ovulation examined its reliability in a fixed-time AI protocol (Candini *et al.* 2001). In this study, control animals were bred at 12, 24 and 36 hours after the onset of estrus, while treated animals received 600 IU of eCG at weaning and 5 mg pLH 72 hours later and were on either a one insemination (24 hours after pLH treatment) or two insemination schedule (24 and 32 hours after pLH treatment). Weaning to estrus interval (WEI) and duration of estrus were shortened with hormonal treatment. Litter size and number born alive was not different among groups, while farrowing rate was lowest in the single insemination group but not significantly different between the double insemination group and control. It is difficult, however, to distinguish the effects of eCG and pLH treatment from each other in this experiment due to the fact that control animals received neither. For example, a decrease in WEI suggests that follicular growth was more rapid in these animals, resulting in an earlier display of estrus. At least part of this result must be due to the effect of eCG, which stimulates follicular growth (Webel and Day 1982). It is important to note the interval from pLH treatment to ovulation, which for both pLH-treated groups was approximately 36 hours, as this has implications for the timing of insemination. The researchers concluded that pLH can be used successfully in a fixed-time AI protocol, but more than one insemination may be required. Nevertheless, with little difference among the pLH-treated groups in farrowing rate and litter size, along with a shorter WEI and duration of estrus, pLH treatment in combination with fixed-time AI would be advantageous in terms of labour requirements alone.

A variation on the protocol outlined above, which would be appropriate for cyclic animals, would be the addition of prostaglandin treatment to ensure all CL's are eliminated prior to ovarian stimulation. Viana *et al.* (2002) tested the timing of prostaglandin administration. Weaned sows were observed for their second estrus after weaning and underwent ultrasound during that estrus to determine the day of ovulation (defined by the researchers as day 0). Animals were then injected on various days with a prostaglandin analogue (7.5 mg Luprostiol), with one group injected on day 12 to 13 (Treatment 1), and others on day 14 (Treatment 2), day 15 (Treatment 3) or day 16 to 17 (Treatment 4). At 24 hours after prostaglandin treatment, animals were injected with 600 IU of eCG, followed 72 hours later by 5 mg pLH. Again ovulation was determined using transrectal ultrasound at six-hour intervals beginning 24 hours after pLH treatment. The results showed that all treatment groups had a similar interval between pLH treatment and ovulation (33 to 42 hours, with an overall average of 39 hours), but Treatment 1 showed the least variation from the average, meaning synchronization was optimal in this group. However, this group had the lowest proportion of animals exhibiting estrus and a longer interval between pLH treatment and onset of estrus. The interval between pLH treatment and estrus in Treatment group 4 averaged -14.4 hours, indicating many animals were exhibiting estrus prior to pLH treatment. This would render the exogenous pLH ineffective and may carry over into the larger variation in the interval between pLH treatment and ovulation in this group. Based on this information, it was concluded by the researchers that administration of prostaglandin in this protocol would be most effective on day 14 to 15 of the estrous cycle, which is equivalent to day 15 to 16 when the day of ovulation is considered day 1 of the estrous cycle.

Two dose-response studies of the Bioniche Lutropin-V pLH product have been published (Gama *et al.* 2005; Viana *et al.* 2005). The first was carried out with prepubertal gilts to establish if pLH could induce puberty and synchronize ovulation. Gama *et al.* (2005) used doses of 1.25, 2.5, and 5 mg of pLH 72 hours after 600 IU of eCG and found that all doses resulted in similar intervals from pLH treatment to ovulation (36 to 38 hours) in those animals that ovulated in response to treatment, with no differences in the occurrence of cystic ovaries or ovulation rate. However, only 60% of the approximately 140 days old animals showed estrus, but the researchers do not indicate if all animals ovulated in response to treatment. Results of this nature in regard to puberty induction can be obtained by other means such as boar exposure, as the majority of gilts are sensitive to the primer pheromones of mature boars at 140 days of age (Estill 1999); therefore, it does not appear that pLH can improve results obtained under other conditions. Furthermore, this approach of initially stimulating follicular growth followed by ovulation induction is not a typical approach to puberty induction. With PG600, follicular growth is stimulated but ovulation occurs naturally. It may not be possible to properly induce puberty using ovulation induction as the animal may not be mature enough to properly respond to the exogenous stimulation, thereby stimulating immature follicles. As well, animals must be at an appropriate age and weight to physiologically respond to the hormone treatment. Recommendations for use of PG600 (Intervet, a combination of 400 IU eCG and 200 IU hCG) for example, indicate that animals should be at least 5.5 months (approximately 165 days) of age and 85 kg (Estill 1999). A study by Pressing *et al.* (1992) showed that prepubertal gilts needed to have an appropriate 'gonadotropin environment' to be able to respond properly to pulsatile GnRH treatments.

Earlier use may induce a response in terms of ovulation but disrupt the gilts' subsequent cycles. This was supported by the finding that if puberty is induced too early with PG600, animals are not likely to continue cycling following the first induced estrus (Paterson *et al.* 1984). Estill (1999) indicates that hormone treatment of this type is more valuable for estrus synchronization than induction of puberty.

The second potency study was reported in sows (Viana *et al.* 2005) and evaluated dosages of 0, 0.625, 1.25, and 2.5 mg of pLH given 56 hours after treatment with 600 IU of eCG at weaning in synchronizing ovulation. Although there was no difference in WEI, duration of estrus, ovulation rate, or number of cystic follicles, only the highest 2.5 mg dose of pLH was able to give significant synchronization of ovulation. Lower dosages of pLH also gave longer intervals from pLH treatment to ovulation (56 to 63 hours) compared to the 2.5 mg treatment (41 hours), which was similar to previous results (Viana *et al.* 2002; Gama *et al.* 2005). Viana *et al.* (2005) concluded that at least 2.5 mg of pLH needs to be used to effectively synchronize ovulation in sows.

Similar research with pLH aimed to establish the synchronizing ability of this product, as well as its usefulness in a fixed-time AI protocol. Results of a dose-response study, evaluating the synchronizing ability of dosages of 0.625, 1.25, 2.5, and 5 mg pLH given 80 hours after 600 IU eCG, confirmed previous research, in that 2.5 mg was the minimum dosage required for consistent results (Bennett-Steward *et al.* 2007). Another set of experiments evaluated the timing of ovulation following pLH treatment and the use of pLH to allow for fixed-time insemination. A pilot study by Cassar *et al.* (2005) evaluated ovulation timing in weaned sows that were treated with 600 IU eCG at weaning followed by 5 mg pLH 80 hours after eCG. Using transrectal ultrasonography at four-hour intervals starting 32 hours after pLH treatment, timing of ovulation was determined. A more extensive trial followed with the same eCG/pLH combination and inseminations at either 36, or 26 and 44, hours after pLH treatment. Other treatment groups included a Control with no hormone treatment, eCG treatment only, and pLH treatment only. Sows in all treatments in which pLH was absent received AM and PM inseminations on the fifth day following weaning. All animals, except those in the eCG/pLH group that received a single insemination, were only inseminated if they exhibited estrus. The pilot study produced similar results to those reported elsewhere, with an average pLH treatment to ovulation interval of 38.2 hours (ranging from 34 to 43 hours). In the more extensive study, there was no difference in the percent of sows exhibiting estrus by day 5, or subsequent fertility parameters, between the eCG only group and Control, illustrating that eCG alone is not effective in increasing pregnancy and farrowing rates. However, as concluded by Cassar *et al.* (2005), this does demonstrate that eCG is not detrimental to fertility. In all treatments, there was no difference in litter size. The pLH treatment alone did increase pregnancy rates and farrowing rates, which were enhanced with the addition of eCG. The authors indicate that this is due to eCG stimulating animals that would typically ovulate later in the estrus period resulting in more optimal inseminations. There was no benefit observed from additional inseminations. This study shows that a fixed-time AI protocol can be employed using pLH and only one insemination, with results being improved on those seen in the control animals of this experiment.

1.4 Hormone Treatment and its Effect on Embryo Quality

There are conflicting opinions on the effect of exogenous hormone treatment on the subsequent litter produced. Prepubertal gilts treated with eCG and hCG may have decreased pregnancy rates (Webel and Day 1982), which could possibly be due to poor quality or immature oocytes being ovulated. It has been suggested that oocyte maturation may be influenced by exogenous hormone treatment, such as hCG (Wiesak *et al.* 1990), which could result in a poorer quality embryo. A study by Ziecik *et al.* (2005) found that hormonally treated gilts had higher proportions of degenerated embryos. On the other hand, some studies suggest that synchronization protocols may be beneficial for embryo survival by decreasing the duration of ovulation, therefore decreasing embryonic heterogeneity (Lambert *et al.* 1991). Although the results of the study by Lambert *et al.* (1991) did show increased embryonic survival following treatment with GnRH or hCG, this increase was not significant. It is possible that conflicting responses to treatment could result, with homogeneity being improved but individual embryo quality being compromised, thereby producing no benefits overall.

It is generally accepted that the more developed embryos of a litter have a better chance of survival (Xie *et al.* 1990). As mentioned previously, Xie *et al.* (1990) were able to associate early ovulating oocytes to more developed embryos and later ovulating oocytes to less developed embryos. Based on this information, one would assume that synchronized ovulation would reduce differences in the variation in embryonic development. This would in turn lead to reduced embryonic loss, because the survival of the less developed embryos is greatly influenced by the presence of more advanced embryos, as demonstrated by Pope *et al.* (1986) using reciprocal embryo transfers. Furthermore, the embryonic loss was not due to the lower viability of these less developed embryos, but the asynchrony with the uterine environment (Wilde *et al.* 1988). Further to this, Pope *et al.* (1988) electrocauterized any follicles smaller that 4 mm at the time of ovulation, and found that this improved embryonic uniformity. However, a study by Soede *et al.* (1992) has rejected this proposition. They determined the duration of ovulation using transrectal ultrasound and compared the resulting embryo diversity to the interval over which ovulation occurred. No relationship was found between these parameters, indicating the rate of development may not be indicative of the time that the oocyte was ovulated. Further to this, it has been suggested that embryonic heterogeneity may be beneficial, as this allows more developed embryos to undergo expansion before others. This in turn inhibits expansion of the lesser developed embryos, thereby eliminating the later crowding issues seen after day 30 of gestation (Geisert and Schmitt, 2002). Nonetheless, it has been suggested that Meishan animals have improved embryonic survival because the litter is more uniform in development (Bazer *et al.* 1988), independent of ovulation rate or follicular heterogeneity (Biggs *et al.* 1993). As well, asynchronous embryonic development may favour implantation of male embryos, thereby altering sex ratios in the surviving litter (Vinsky *et al.* 2006; Foxcroft *et al.* 2007). Recent evidence also suggests that a subpopulation of animals exist that produce heterogeneous litters, regardless of any treatment imposed prior to ovulation, and these animals are predicted to carry litters with poor embryonic survival to day 30 of gestation (Vinsky *et al.* 2007).

A study by Tain *et al.* (2001) involving rats, looked at the effect of eCG alone, and in combination with hCG, on the embryo by examining early embryonic morphology (zona pellucida integrity, blastomere regularity, distribution of granules in the cytoplasm, and the amount of fragmentation and degeneration) and pregnancy rates. Excessive doses of eCG decreased pregnancy rates, but early pregnancy rates were improved when eCG was used in combination with hCG. High eCG doses also had a detrimental effect on embryo morphology. When hCG was included, improvements were seen in early embryo morphology but, in the long term, no significant improvements were seen.

With respect to swine, a recent study used a treatment of eCG followed by pLH to induce estrus in pre-pubertal gilts, and then compared the effect of an additional hormonal treatment on synchronization and embryo viability (do Lago *et al.* 2005). Firstly, this study found that the second hormonal treatment resulted in more animals showing estrus within a tighter interval. Secondly, when embryo viability was examined morphologically (uniformity of the perivitelline space and distribution of the blastomeres), it was noticed that the number of viable embryos was reduced in the hormone treatment group, although ovulation rate was the same.

The effect of hormonally inducing estrus on embryo quality was also studied by Holtz *et al.* (1999). This study compared a control group of prepubertal gilts inseminated on their first spontaneous estrus to treatment groups in which puberty was induced with PG600 and then animals were inseminated on their first or second estrus following PG600 treatment. The treatment group inseminated on their first estrus following induction had a higher rate of return and lower litter sizes in their first litter than those inseminated on their second estrus. The poor first litter performance of the animals inseminated following an induced estrus does not seem to continue into subsequent litters resulting from cycles that were not pharmacologically influenced. Differences in litter size disappear by the second litter (Holtz *et al.* 1999); there was also no difference between the control animals and induced animals inseminated on their second estrus.

Bolamba *et al.* (1996) looked at the effect of FSH, eCG, or a combination of eCG and hCG on oocyte quality. Pronuclear formation was lower in controls and FSH-treated animals but there was no effect on rate of polyspermy and the number of 'good' oocytes was actually increased with FSH treatment. A follow-up experiment exposed prepubertal gilts to different dosages of FSH or hCG, and examined the competency of the resulting oocytes/embryos after *in vitro* maturation/*in vitro* fertilization (IVM/IVF) (Bolamba and Sirard 2000). Polyspermy rates and pronuclear formation were unaffected by treatment, but embryo development rates were slightly improved with a treatment of 8 mg of FSH given three times.

A study by Lui *et al.* (2003) looked at rates of apoptosis in follicles following eCG treatment, to study follicular development and to see if treatment can 'rescue' follicles. Their results indicate that rates of apoptosis are reduced in both mature and immature gilts treated with eCG; however, without further stimulation in immature animals, rates of apoptosis eventually returned to control levels. If follicles are rescued and then ovulate, this brings into question the quality of the oocytes from those rescued follicles.

Another study looking at the effect of superovulation on embryonic development at day 5 found that there was no difference in rate of development between superovulated animals (high dose of eCG) versus non-superovulated animals (lower eCG dosage) (Hazeleger *et al.* 2000). There was also no significant difference in blastocyst diameter or pregnancy rates between the two eCG treatments after embryo transfer. Conversely, Hazeleger *et al.* (2000) did find that blastocysts from animals treated with 1500 IU eCG had fewer total cells than animals treated with 1000 IU eCG.

1.5 Morphological Indicators of Embryo Quality

There is the potential for high embryonic loss in the first 30 days of gestation (Geisert and Schmitt 2002). Due to the impact this has on litter size, it is important to understand the mechanisms by which this occurs. One major factor is the synchrony of the uterus in relation to the embryo, which relates back to the duration of ovulation (Geisert and Schmitt 2002). However, the quality of the embryo itself is also of great importance.

The embryo has a strong influence on its environment through its production of estrogen (Geisert and Schmitt 2002). As a result, it is important for the less advanced embryos of a litter to be developmentally competent enough to deal with the changing environment created by the more developed embryos.

Many approaches have been used to determine embryo maturity and possibly quality. In general, morphological characteristics have been used to evaluate embryo quality. In very early embryos, pronuclear formation and rates of polyspermy have been used to determine oocyte/embryo quality (Bolamba and Sirard 2000). Oocytes have also been rated on the degree of expansion of the cumulus cells and the status of nuclear maturation (Zak *et al.* 1997). Of course it is relatively straightforward to distinguish maturity when embryos are at different stages of development. In an early study, where 3- to 9-day embryos were collected and examined, few embryos could be considered poor when looked at morphologically, except those that were clearly unfertilized or degenerated; however, it was admitted that such superficial examination may not be reliable (Polge 1982). Further morphological evaluations, such as cell counting and ratios of cell populations within the developing embryo have also been used. In the prolific Meishan breed, the size of the inner cell mass (ICM) is similar to other breeds but the number of cells in the trophectoderm (TE) is lower (Rivera *et al.* 1996). For embryo quality evaluation in cattle, embryos have begun to be assessed by looking at the ratio between ICM and TE and by counting the number of apoptotic cells (Fouladi-Nashta *et al.* 2005). The number of ICM cells appears to be an important factor for blastocyst viability, and significant loss of ICM cells can lead to delay of development and failure of the embryo to implant (Tam 1988). Differential staining methods have been developed to help identify ICM and TE specific cells. One method appears to be a particularly rapid and inexpensive way of accomplishing this. This involves permeablizing the TE cells, staining the nuclei with propidium iodide, and then following with a 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) stain to stain all cell nuclei (Thouas *et al.* 2001). Fouladi-Nashta *et al.* (2005) further modified this by adding terminal dexoynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) staining to label apoptotic cells.

Fouladi-Nashta *et al.* (2005) suggest that embryos with increased numbers of both ICM and TE were associated with embryos that are developmentally more competent.

The importance of detecting apoptotic cells is further described in a review by Jurisicova and Acton (2004), which highlights the major problem with manipulating human embryos in *in vitro* conditions. The difficulty is that even embryos that are able to develop to the blastocyst stage are still vulnerable to apoptosis and degeneration. Embryos that typically do not achieve the blastocyst stage have arrested growth or embryo fragmentation. Cell death is regulated by genes from many families, and the fate of the cell will be decided by the ability of cell death suppressors to control cell death inducers. This review suggests that there is an inherent level of expression of these genes that predisposes the embryo to either apoptosis or survival. It also indicates that the ability of embryos to undergo proper cleavage without fragmentation is influenced by maternal effects, as well as the presence of maternal factors that influence the ability of the embryo to implant (Jurisicova and Acton 2004). Therefore, the rate of apoptosis in embryos could be an indicator of the embryo 'quality' and its ability to remain viable. Also, the incidence of apoptosis may be a result of maternal effects, highlighting the effect that treatments applied to the mother could have on embryo quality.

A similar differential staining technique, with TUNEL staining being omitted, has been applied to porcine embryos in a study by Kim *et al.* (2004) to evaluate the effects of different culture media. It was found that faster rates of development were associated with increased numbers of ICM and TE cells, and, therefore, total cells (Kim *et al.* 2004). Due to the increase in both types of cells, the ratio between ICM and total cells for both fast and slow developing embryos did not differ. Total cell counting has also been used on porcine embryos using Giemsa staining to evaluate quality (Hazeleger *et al.* 2000).

Another method of evaluating embryo quality has been to expose them to a period of *in vitro* culture following collection and then score rates of development from 2- and 4-cells to morula and blastocyst stages. A study by Almeida *et al.* (2001) used this indicator to evaluate the effect of nutrition during the estrous cycle on embryo quality. This approach would allow observations of individual embryos over time. However, *in vitro* porcine culture systems lag behind those developed in cattle and *in vitro* manipulated embryos tend to have poorer development when compared to development of embryos *in vivo* (Niemann and Rath 2001). Therefore, differences in development may be confounded by *in vitro* culture and these differences may not be attributable to treatment effects.

The degree of expansion of the blastocyst may be another indicator of developmental competence. Typically, blastocysts range from 0.5 to 1 mm in diameter at hatching (day 6 to 7), and increase to 2 to 6 mm at day 10 prior to undergoing elongation (Bazer *et al.* 1982). As mentioned previously, Hazeleger *et al.* (2000) found that blastocyst diameter could be a valuable parameter in determining embryo quality, with larger diameters indicating a well-functioning embryo. Further to this, Fujino *et al.* (2006) tried to determine the relationship between diameter and the stage of development 6 to 7 days following hCG treatment. They found that early blastocysts had diameters of 225 μm or less, expanded blastocysts tended to have a diameter greater than 225 μm, and hatching tended to occur when the diameter reached 250 μm or more (Fujino *et al.* 2006). These researchers indicate a positive correlation between cell counts and embryo diameter has previously been reported.

1.6 Gene Expression and Embryo Quality

Molecular approaches to evaluating embryo quality have also been attempted. The polysaccharide hyaluronic acid (HA), a component of the extracellular matrix, is an important factor in cumulus cell expansion during ovulation (Kimura *et al.* 2002). LH release is an important signal in initiating this expansion, hence its importance for stimulating ovulation (Kimura *et al.* 2002). HA has also been found to be in high concentrations in the uterine fluid of mice, swine, cattle, and humans, and promotes early development of porcine and bovine embryos *in vitro* (Furnus *et al.* 2003). The CD44 receptor can bind to HA, and it has been found to be expressed on the surface and in the cytoplasm of human and bovine oocytes and embryos (Furnus *et al.* 2003). Furthermore, bovine embryos from 2-cell stage or more have been shown to internalize tagged HA (Furnus *et al.* 2003). Kimura *et al.* (2002) suggest that the porcine oocyte secretes factors to promote HA production by the cumulus cells, and it has been suggested that these factors are HA synthases similar to those that have already been identified in other species. HA is also considered to promote cell proliferation and CD44 can bind certain growth factors, influencing their biological activity (Kano *et al.* 1998). Therefore, expression of CD44 by the porcine oocyte or embryo has been considered as a potential indicator of embryo quality.

When *in vitro*-derived porcine embryos were cultured with HA, a greater percentage of embryos developed to the blastocyst stage compared to other glycosaminoglycans (GAG's), and those blastocysts tended to have higher mean cell numbers, although the difference was not significant (Kano *et al.* 1998). Using reverse transcriptase polymerase chain reaction (RT-PCR) analysis to detect mRNA expression, Kimura *et al.* (2002) were able to show that the porcine oocyte expresses has3 (a HA synthase). Using immunofluorescence, CD44 was found to be present at the junctions between the cumulus cells and the oocyte (Kimura *et al.* 2002). The authors suggest that the presence of the oocyte promoted cumulus cell expansion, possibly by stimulating has2 expression by the cumulus cells (Kimura *et al.* 2002). No CD44 mRNA expression was seen in the oocyte, but immunostaining of immature oocytes was positive for CD44 (Kimura *et al.* 2002). A later study by Kim *et al.* (2005), however, was able to detect CD44 mRNA in mature porcine oocytes as well as all early embryonic stages, including blastocysts. However, in contrast to the study previously discussed, HA in culture did not enhance embryo development, and in fact, high concentrations of HA had a detrimental effect on blastocyst development (Kim *et al.* 2005). A moderate concentration of HA was able to increase total cell numbers, but did not influence the number of cells in the ICM (Kim *et al.* 2005). A study by Toyokawa *et al.* (2005) focused on HA alone and found that HA was able to promote development of parthenogenetic embryos and CD44 expression was detected on the embryonic cell membranes throughout early development. This study went on to block the binding of HA with a CD44 antibody and in doing so, interrupted development at the four cell stage (Toyokawa *et al.* 2005). With regards to other GAG's, work has been conducted to evaluate the effects of heparin, which binds to the HIP receptor that is also found in all early embryonic stages similarly to CD44. Kim *et al.*

(2005) found that culture containing heparin actually gave better results than HA, which is in direct contrast to Kano *et al.*'s (1998) study which found that heparin had no effect on embryo development. Although it would appear that GAG's, and in particular HA, play a role in embryonic development, their role is not fully defined and the potential use of CD44 expression as a predictor of embryo quality is unclear.

Estrogen production by pig embryos would presumably be important to embryonic survival, as it is the porcine signal for maternal recognition of pregnancy (Spencer *et al.* 2004). However, in Meishan pigs which tend to be more prolific, embryos actually produce less estrogen. This is thought to be beneficial because the embryo induces less change in the uterine environment, thereby reducing the effect of more advanced embryos on less developed ones (Geisert and Schmitt 2002). Regardless, stereoidogenic gene expression is irrelevant to the evaluation of embryo quality in early embryos as estrogen production does not begin until approximately day 11 to 12 of gestation (Geisert *et al.* 1990). The expression of various growth factors is influenced by estrogen production, which in turn influences the embryo in preparation for implantation (Spencer *et al.* 2004). The expression of receptors for these growth hormones by the embryo may also be of interest in regards to embryo quality, but again, this occurs too late to evaluate early embryos.

Insulin-like growth factor-I (IGF-I) production by the uterus appears to peak at a time when estrogen production by the embryo is high, highlighting a possible function for IGF-I in promoting embryo development (Green *et al.* 1995). IGF-I receptor (IGF-IR) was found to be constitutively expressed by day 8 to 15 embryos, and IGF-I treatment was only able to increase P450 aromatase activity in filamentous embryos, not spherical blastocysts (Green *et al.* 1995). Overall, Green *et al.* (1995) suggest that estrogens from embryos stimulate IGF-I production by the uterus, which in turn leads to increased expression of the stereoidogenic enzyme P450 aromatase in the embryo, so IGF-IR expression appears to be beneficial for the conceptus. However, with IGF-IR being constitutively expressed, it would not be a reliable indicator of differences among embryos, and therefore, it would not be a consistent way of evaluating embryo quality.

Also, the stimulatory effect of IGF-I is not seen until the embryo is filamentous, so evaluating IGF-IR expression would not be advantageous at earlier stages of development. However, as the area of functional genomics moves forward and the gene expression patterns of the developing embryo are defined, it is expected that molecular markers of embryo quality will be characterized.

1.7 Conclusions

As reviewed, there have been many attempts to synchronize ovulation and estrus in swine, with varying results. With regard to technologies that take advantage of this synchronization, the use of eCG followed by hCG has emerged as the primary method of inducing porcine ovulation, but synchronization is not always consistent. Furthermore, there are implications of exogenous hormone treatment for litter heterogeneity and embryo quality that have not been concretely established. Further to this, ways of evaluating these parameters are under development and are also uncertain in their reliability. With the development of a pLH product, there is the possibility of addressing the problem of variation among animals in their response to exogenous hormone treatment. As well, measures of embryonic quality require further study and methods of determining viability of the very early embryo would be invaluable for protocols involving embryo transfer. With this in mind, the objectives of the research conducted and presented in the following chapters of this thesis were to: 1) establish if pLH is appropriate for efficient and reliable induction of ovulation in cyclic gilts; 2) determine its effects, if any, on the embryos resulting from the induced ovulations; 3) investigate approaches to evaluating porcine embryo quality early in development.

1.8 References

Almeida, F.R.C.L., Mao, J., Novak, S., Cosgrove, J.R., Foxcroft, G.R. 2001. Effects of different patterns of feed restriction and insulin treatment during the luteal phase on reproductive, metabolic, and endocrine parameters in cyclic gilts. *J. Anim. Sci.* 79:200–212.

Ambrose, J.D., Kastelic, J.P., Rajamahendran, R., Aali, M., Dinn, N. 2005. Progesterone (CIDR)-based timed AI protocols using GnRH, porcine LH or estradiol cypionate for dairy heifers: Ovarian and endocrine responses and pregnancy rates. *Theriogenology.* 64:1457–1474.

Baer, C., Bilkei, G. 2004. The effect of intravaginal applied GnRH-agonist on the time of ovulation and subsequent reproductive performance of weaned multiparous sows. *Reprod. Dom. Anim.* 39:293–297.

Baruselli, P.S., de Sa Filho, M.F., Martins, C.M., Nasser, L.F., Nogueria, M.F., Barros, C.M., Bo, G.A. 2006. Superovulation and embryo transfer in *Bos indicus* cattle. *Theriogenology.* 65:77–88.

Bazer, F.W., Geisert, R.D., Thatcher, W.W., Roberts, R.M. 1982. The establishment and maintenance of pregnancy. In Control of Pig Reproduction. Cole, D.J.A, Foxcroft, G.R. Eds. pp. 227-252.

Bazer, F.W., Thatcher, W.W., Martinat-Botte, F., Terqui, M. 1988. Conceptus development in Large White and prolific Chinese Meishan pigs. *J. Reprod. Fert.* 84:37-42.

Bennett-Steward, K., Cassar, G., Plante, C., Friendship, R.M., Zak, L. 2007. Ovulation induction protocol using equine chorionic gonadotropin and porcine luteinizing hormone in weaned sows. *J. Swine Health Prod.* 15: 194-197.

Biggs, C., Tilton, J.E., Craigon, J., Foxcroft, G.R., Ashworth, C.J., Hunter, M.G. 1993. Comparisons of follicular heterogeneity and ovarian characteristics in Meishan and Large-White hybrid pigs. *J. Reprod. Fert.* 97:263-269.

Bo, G.A., Baruselli, P.S., Chesta, P.M., Martins, C.M. 2006. The timing of ovulation and insemination schedules in superstimulated cattle. *Theriogenology*. 65:89–101.

Bo, G.A., Baruselli, P.S., Moreno, D., Cutaia, L., Caccia, M., Tribulo, R., Tribulo, H., Mapeltoft, R.J. 2002. The control of follicular wave development for self-appointed embryo transfer programs in cattle. *Theriogenology.* 57:53-57.

Bolamba, D., Dubuc, A., Dufour, J. J., Sirard, M- A. 1996. Effects of gonadotropin treatment on ovarian follicle growth, oocyte quality, and in vitro fertilization of oocytes in prepubertal gilts. *Theriogenology.* 46:717-726.

Bolamba, D., Sirard, M.-A. 2000. Ovulation and follicular growth in gonadotropin-treated gilts followed by in vitro fertilization and development of their oocytes. *Theriogenology.* 53:1421-1437.

Boland, M.P., Crosby, T.F., Gordon, I. 1978. Morphological normality of cattle embryos following superovulation using PMSG. *Theriogenology.* 10:175-180.

Brussow, K.P., Jochle, W., Huhn, U. 1996. Control of ovulation with a GnRH analog in gilts and sows. *Theriogenology.* 46:925-934.

Candini, P.H., Moretti, A.S., Silveria P.R.S., Zanella, E., Viana, C.H.C., Santos, I.C.C. 2001. Utilization of a protocol of synchronization of ovulation, induced by porcine LH, to perform insemination programs in fixed time, with one or two inseminations per sow. Sixth International Conference on Pig Reproduction, Columbia MO. pp. 99.

Cassar, G., Kirkwood, R.N., Bennett-Steward, K., Friendship, R.M. 2005. Towards timed single insemination in sows. Proceedings of the 36th Annual Meeting of the American Association of Swine Veterinarians, Toronto, ON. pp. 353-356.

D'Alessandro, A.G., Martemucchi, G., Colonna, M.A., Borghese, A., Terzano, M.G., Bellitti, A. 2001. Superovulation in ewes by a single injection of pFSH dissolved in polyvinylpyrrolidone (PVP): effects of PVP molecular weight, concentration and schedule of treatment. *Anim. Reprod. Sci.* 65:255-264.

Demoustier M.M., Backers, J-Fr., Van Der Zwalmen, P., Closset, J., Gillard, J-L., Ectors Fr. 1988. Determination of porcine plasma follitropin levels during superovulation treatment in cows. *Theriogenology.* 30:379-386.

De Rensis, F., Benedetti, S., Silva, P., Kirkwood, R.N. 2003. Fertility of sows following artificial insemination at a gonadotrophin-induced estrus coincident with weaning. *Anim. Repro. Sci.* 76:245–250.

do Lago, V., Vianna, W.L., Gama, R.D., de Campos Rosseto, A., Pinese, M.E., de Sant'Anna Moretti, A. 2005. Second oestrus synchronization and precocious embryo viability after puberty induction in gilts by the use of gonadotrophin treatment. *Reprod. Dom. Anim.* 40:141–144.

Dufau, M.L. 1998. The luteinizing hormone receptor. *Ann. Rev. Physiol.* 60:461-496.

Ellendorff, F., Parvizi, N. 1982. The central nervous system and the control of pituitary hormone release in the pig. In Control of Pig Reproduction. Cole, D.J.A, Foxcroft, G.R. Eds. pp. 179-195.

Elsaesser, F. 1982. Endocrine control of sexual maturation in the female pig and sexual differentiation of the stimulatory oestrogen feedback mechanism. In Control of Pig Reproduction. Cole, D.J.A, Foxcroft, G.R. Eds. pp. 93-116.

Estill, C.T. 1999. Current concepts in estrus synchronization in swine. Proceedings of the American Society of Animal Science. Southern Section ASAS Meeting, Memphis, TN. E38.

Estill, C.T., Britt, J.H., Gadsby, J.E. 1993. Repeated administration of Prostaglandin F during the early luteal phase causes premature luteolysis in the pig. *Biol. Repro.* 49:181-185.

Foxcroft, G.R, Aherne, F.X, Clowes, E.C, Miller, H., and Zak, L. 1995. Sow Fertility: The role of suckling inhibition and metabolic status. In Animal Science Research and

Development: Moving Towards a New Century. Ivan, M. ed. Center of Food and Animal Research, Ottawa, Canada. pp. 377-393.

Foxcroft, G.R., Van de Wiel, D.F.M. 1982. Endocrine control of the oestrous cycle. In Control of Pig Reproduction. Cole, D.J.A, Foxcroft, G.R. Eds. pp. 161-177.

Foxcroft, G.R., Vinsky, M.D., Paradis, F., Tse, W-Y., Town, S.C., Putman, C.T., Dyck, M.K., Dixon, W.T. 2007. Macro-environment effects on oocytes and embryos in swine. *Theriogenology.* Epub.

Fouladi-Nashta, A.A., Alberio, R., Kafi, M., Nicholas, B., Campbell, K.H.S., Webb, R. 2005. Differential staining combined with TUNEL labeling to detect apoptosis in preimplantation bovine embryos. Reproductive BioMedicine Online. 10:497-502.

Fujino, Y., Nakamura, Y., Kobayashi, H., Kikuchi, K. 2006. Relationship between time elapsed after human chorionic gonadotropin administration and developmental stage in porcine embryos collected from prepubertal gilts. *J. Reprod. Dev.* 52:267-275.

Furnus, C.C., Valcarcel, A., Dulout, F.N., Lı´a Errecalde, A. 2003. The hyaluronic acid receptor (CD44) is expressed in bovine oocytes and early stage embryos. *Theriogenology.* 60:1633-1644.

Gama, R.D., Vianna, W.L., Pinese, M.E., de Campos Rosseto, A., de Sant'Anna Moretti, A. 2005. Different doses of porcine luteinizing hormone in precocious puberty induction in gilts. *Reprod. Dom. Anim.* 40:433-435.

Garcia-Campayo, V., Boime, I. 2001. Novel recombinant gonadotropins. *Trends Endocrinol. Metab.* 12:72-77.

Geisert, R.D., Schmitt, R.A.M. 2002. Early embryonic survival in the pig: Can it be improved? *J. Anim. Sci.* (E. Supplement 1) 80:E54-E65.

Geisert, R.D., Zavy, M.T., Moffatt, R.J., Blair, R.M., Yellin, T. 1990. Embryonic steroids and the establishment of pregnancy in pigs. *J. Reprod. Fertil. Suppl.* 40:293-305.

Goulding, D., Williams, D.H., Roche, J.F., Boland, M.P. 1991. Superovulation in heifers using either pregnant mare's serum gonadotrophin or follicle stimulating hormone during the mid luteal stage of the estrous cycle. *Theriogenology.* 36:949-958.

Green, M.L., Simmen, R.C.M., Simmen, F.A. 1995. Developmental regulation of stereoidogenic enzyme gene expression in the periimplantation porcine conceptus: A paracrine role for insulin-like growth factor-I. *Endocrinology.* 136: 3961-3970.

Guthrie, H.D. 2005. The follicular phase in pigs: Follicle populations, circulating hormones, follicle factors, and oocytes. *J. Anim. Sci.* 83(Suppl.):E79-89.

Guthrie, H.D., Bolt, D.J. 1985. Pituitary and ovarian hormone secretion and ovulation in gilts injected with gonadotropins during and after oral administration of progesterone agonist (Altrenogest). *Biol. Repro.* 33:679-689.

Hazeleger, W., Bouwman, E.G., Noordhuizen, J.P.T.M., Kemp, B. 2000. Effect of superovulation induction on embryonic development on day 5 and subsequent development and survival after nonsurgical embryo transfer in pigs. *Theriogenology.* 53:1063-1070.

Hazeleger, W., Kemp, B., Soede, N.M., Noordhuizen, J.P.T.M., van der Lende, T. 1994a. Transcervical embryo collection and reproductive performance of sows with resectioned uteri. *Reprod. Dom. Anim.* 29:38-45.

Hazeleger, W., Kemp, B. 1994b. Farrowing rate and litter size after transcervical embryo transfer in sows. *Reprod. Dom. Anim.* 29:481-487.

Holtz, W., Schmidt-Baulain, R., Welp, C., Wallenhorst, Chr. K. 1999. Effect of insemination of estrus-induced prepuberal gilts on ensuing reproductive performance and body weight. *Anim. Repro. Sci.* 57:177-183.

Hughes, P.E. 1982. Factors affecting the natural attainment of puberty in the gilt. In Control of Pig Reproduction. Cole, D.J.A, Foxcroft, G.R. Eds. pp. 117-138.

Hunter, M.G. 2000. Oocyte maturation and ovum quality in pigs. *Reviews of Reproduction.* 5:122-130.

Hunter, R.H.F. 1967. Porcine ovulation after injection of human chorionic gonadotrophin. *Vet. Rec.* 81:21-23.

Jurisicova, A., Acton, B. 2004. Deadly decisions: the role of genes regulating programmed cell death in human preimplantation embryo development. *Reproduction.* 128:281–291.

Kano, K., Miyano, T., Kato, S. 1998. Effects of glycosaminoglycans on the development of in vitro-matured and -fertilized porcine oocytes to the blastocyst stage in vitro. *Biol. Repro.* 58:1226-1232.

Kim, H-s., Lee, G-s., Hyun, S-h., Lee, S-h., Nam, D-h., Jeong, Y-w., Kim, S., Kang, S-k., Lee, B-c., Hwang, W-s. 2004. Improved in vitro development of porcine embryos with different energy substrates and serum. *Theriogenology.* 61:1381-1393.

Kim, H-s., Lee, G-s., Hyun, S-h., Nam, D-h., Lee, S-h., Jeong, Y-W., Kim, S., Kim, J-h., Kang, S-k., Lee, B-c., Hwang, W-s. 2005. Embryotropic effect of glycosaminoglycans and receptors in development of porcine pre-implantation embryos. *Theriogenology.* 63:1167-1180.

Kimura, N., Konno, Y., Miyoshi, K., Matsumoto, H., Sato, E. 2002. Expression of hyaluronan synthases and CD44 messenger RNAs in porcine cumulus-oocyte complexes during in vitro maturation. *Biol. Repro.* 66:707-717.

Knox, R., Willenburg, K., Rodriguez-Zas, S., Gregor D., Swanson, M. 2003. Novel hormone treatment may lead to fixed-time AI. National Hog Farmer. November 15. pp. 20

Lambert, E., Williams, D.H., Lynch, P.B., Hanrahan, T.J., McGeady, T.A., Austin, F.H., Boland, M.P., Roche, J.F. 1991. The effects of administration of gonadotrophins and estrogens on prenatal survival in gilts. *Theriogenology.* 36:77-85.

Langendijk, P., Soede, N.M., Kemp, B. 2005. Uterine activity, sperm transport, and the role of boar stimuli around insemination in sows. *Theriogenology.* 63:500-513.

Larson, J.E., Lamb, G.C, Stevenson, J.S., Johnson, S.K., Day, M.L., Geary, T.W., Kesler, D.J., DeJarnette, J.M., Schrick, F.N., DiCostanzo, A., Arseneau, J.D. 2006. Synchronization of estrus in suckled beef cows for detected estrus and artificial insemination and timed artificial insemination using gonadotropin-releasing hormone, prostaglandin F2*a*, and progesterone. *J. Anim. Sci.* 84:332–342.

Liu, Z.H., Yue, K.Z., Ma, S.F., Sun, X.S., Tan, J.H. 2003. Effects of pregnant mare serum gonadotropin (eCG) on follicle development and granulosa-cell apoptosis in the pig. *Theriogenology.* 59:775-785.

Lopez-Gatius, F., Santolaria, P., Yaniz, J.L., Hunter, R.H.F. 2004. Progesterone supplementation during the early fetal period reduces pregnancy loss in high-yielding dairy cattle. *Theriogenology.* 62:1529-1535.

Martinez, M.F., Adams, G.P., Bergfelt, D.R., Kastelic, J.P., Mapletoft, R.J. 1999. Effect of LH or GnRH on the dominant follicle of the first follicular wave in beef heifers. *Anim. Repro. Sci.* 57:23–33.

Niemann, H., Rath, D. 2001. Progress in reproductive biotechnology in swine. *Theriogenology.* 56:1291-1304.

Paterson, A.M., Pearce, G.P., Foxcroft, G.R. 1984. Reproductive performance of gilts induced into puberty with oestradiol benzoate or a combination of pregnant mare's serum gonadotropin and human chorionic gonadotropin. *Anim. Prod.* 38:121-128.

Patterson, J.L., Ball, R.O., Willis, H.J., Aherne, F.X., Foxcroft, G.R. 2002a. The effect of lean growth rate on puberty attainment in gilts. *J. Anim. Sci.* 80:1299-1310.

Patterson, J.L., Willis, H.J., Kirkwood, R.N., Foxcroft, G.R. 2002b. Impact of boar exposure on puberty attainment and breeding outcomes in gilts. *Theriogenology.* 57:2015-2025.

Pereira, C.Z., Vasconcelos, J.L.M., Berto, D.A., Vieira, L.N., Morais, M.N.P. 2001. Synchronization of ovulation and timed artificial inseminations in sows in an early weaning system. Sixth International Conference on Pig Reproduction, Columbia MO. pp. 122.

Polge, C. 1982. Embryo transplantation and preservation. In Control of Pig Reproduction. Cole, D.J.A, Foxcroft, G.R. Eds. pp. 277-291.

Pope, W.F., Lawyer, M.S., Nara, B.S., First, N.L. 1986. Effect of asynchronous superinduction on embryo survival and range of blastocyst development in swine. *Biol. Repro.* 35:133-137.

Pope, W.F., Wilde, M.H., Xie, S. 1988. Effect of electrocautery of nonovulated day 1 follicles on subsequent morphological variation among day 11 porcine embryos. *Biol. Repro.* 39:882-887.

Pressing, A., Dial, G. D., Esbenshade, K. L., Stroud, C. M. 1992. Hourly administration of GnRH to prepubertal gilts: endocrine and ovulatory responses from 70 to 190 days of age. *J. Anim. Sci.* 70:232-242.

Pressing, A.L., Dial, G. D., Stroud, C. M., Almond G.W., Robison, O.W. 1987. Prostaglandin-induced abortion in swine: endocrine changes and influence on subsequent reproductive activity. *Am. J. Vet. Res.* 48:45-50.

Rivera, R.M., Youngs, C.R., Ford, S.P. 1996. A comparison of the number of inner cell mass and trophectoderm cells of preimplantation Meishan and Yorkshire pig embryos at similar developmental stages. *J. Reprod. Fertil.* 106:111-116.

Roski, K. 2004. Ovulatory and reproductive characteristics of sows treated with an intravaginal GnRH agonist gel. Master's Thesis, North Carolina State University.

Soede, N. M., Noordhuizen, J.P.T.M., Kemp, B. 1992. The duration of ovulation in pigs, studied by transrectal ultrasonography, is not related to early embryonic diversity. *Theriogenology.* 38:653-666.

Spencer, T.E., Burghardt, R.C., Johnson, G.A., Bazer, F.W. 2004. Conceptus signals for establishment and maintenance of pregnancy. *Anim. Repro. Sci.* 82-83:537-550.

Staigmiller, R.B., MacNeil, M.D., Bellows, R.A., Short, R.E., Phelps, D.A. 1995. The effect of estrus synchronization scheme, injection protocol and large ovarian follicle in response to superovulation in beef heifers. *Theriogenology.* 43:823-834.

Stone, B.A., Heap, P.A., Seamark, R.F. 1987. Changes in peripheral progestagen levels in early pregnant gilts following injection of human chorionic gonadotrophin. *J. Endocrinol.* 115(1):161-167.

Tain, C.F., Goh, H.H.V., Ng, S.C. 2001. Dose–response effects of equine chorionic gonadotrophin (eCG) and human chorionic gonadotrophin (hCG) on early embryonic development and viable pregnancy rate in rats. *Reproduction.* 122:283–287.

Tam, P.P.L. 1988. Postimplantation development of mitomycin c-treated mouse blastocysts. *Teratology.* 37:205-212

Thouas, G., Korfiatis, N.A., French, A.J., Jones, G.M., Trounson, A.O. 2001. Simplified technique for differential staining of inner cell mass and trophectoderm cells of mouse and bovine blastocysts. Reproductive BioMedicine Online. 3:25-29.

Tilton, J.E, Foxcroft, G.R., Ziecik, A.J., Coombs, S.L., Williams, G.L. 1982. Time of the preovulatory LH surge in the gilt and sow relative to the onset of behavioral estrus. *Theriogenology.* 18:227-236.

Toyokawa, K., Harayama, H., Miyake, M. 2005. Exogenous hyaluronic acid enhances porcine parthenogenetic embryo development in vitro possibly mediated by CD44. *Theriogenology.* 64:378–392.

Tummaruk, P., Tantasuparuk, W., Techakumphu, M., Kunavongkrit, A. 2007. Age, body weight and backfat thickness at first observed oestrus in crossbred Landrace x Yorkshire gilts, seasonal variations and their influence on subsequent reproductive performance. *Anim. Reprod. Sci.* 99:167-181.

Van de Wiel, D.F.M., Erkens, J., Koops, W., Vos, E., Van Landeghem, A.A.J. 1981. Periestrous and midluteal time courses of circulating LH, FSH, prolactin, estradiol-17β and progesterone in the domestic pig. *Biol. Reprod.* 24:223-233.

Viana, C.H.C., Candini, P.H., Gama, R.D., Carbone, A., Santos, I.C.C., Vianna, W.L., Barnabe, R.C. 2002. Evaluation of a synchronization of ovulation protocol in cyclic sows. Proceedings of the International Pig Veterinary Congress, Ames, Iowa #678.

Viana, C.H.C., Castricini, E.S.C., Santos, I.C.C. 2005. Determination of the minimum porcine LH dose on synchronization of ovulation in sows. Presented at the International Congress on Pig Production, Amsterdam.

Vinsky, M.D., Novak, S., Dixon, W.T., Dyck, M.K., Foxcroft, G.R. 2006. Nutritional restriction in lactating primiparous sows selectively affects female embryo survival and overall litter development. *Reprod. Fertil. Dev.* 18:347-55.

Vinsky, M.D., Paradis, F., Dixon, W.T., Dyck, M.K., Foxcroft, G.R. 2007. Ontogeny of metabolic effects on embryonic development in lactating and weaned primiparous sows. *Reprod. Fertil. Dev.* 19:603-611.

Walsh, J.H., Mantovani, R., Duby, R.T., Overstrom, E.W., Dobrinsky, J.R., Enright, W.J., Roche, J.F., Boland, M.P. 1993. The effects of once or twice daily injections of pFSH on superovulatory response in heifers. *Theriogenology.* 40:313-321.

Webel, S.K., Day, B.N. 1982. The control of ovulation. In Control of Pig Reproduction. Cole, D.J.A, Foxcroft, G.R. Eds. pp. 197-210.

Wiesak, T., Hunter, M.G., Foxcroft, G.R. 1990. Differences in follicular morphology, steroidogenesis and oocyte maturation in naturally cyclic and PMSG/hCG-treated prepubertal gilts. *J. Reprod. Fert.* 89:633-41.

Wilde, M.H., Xie, S., Day, M.L., Pope, W.F. 1988. Survival of small and large littermate blastocysts in swine after synchronous and asynchronous transfer procedures. *Theriogenology.* 30:1069-1074.

Wood, C. M., Kornegay, E. T., Shipley, C. F. 1992. Efficacy of altrenogest in synchronizing estrus in two swine breeding programs and effects on subsequent reproductive performance of sows. *J. Anim. Sci.* 70:1357-1364.

Xie, S., Broermann, D.M., Nephew, K.P., Geisert, R.D., Pope, W.F. 1990. Ovulation and early embryogenesis in swine. *Biol. Repro.* 43:236-240.

Zak, L.J., Xu, X., Hardin, R.T., Foxcroft, G.R. 1997. Impact of different patterns of feed intake during lactation in the primiparous sow on follicular development and oocyte maturation. *J. Reprod. Fert.* 110:99-106.

Ziecik, A.J. 2002. Old, new and the newest concepts of inhibition of luteolysis during early pregnancy in pig. *Domestic Animal Endocrinology.* 23:265–275.

Ziecik, A.J., Biallowicz, M., Kaczmarek, M., Demianowicz, W., Rioperez, J., Wasielak, M., Bogacki, M. 2005. Influence of estrus synchronization of prepubertal gilts on embryo quality. *J. Reprod. Dev.* 51:379-384.

Chapter 2

ENDOCRINE RESPONSE AND SYNCHRONIZATION OF OVULATION IN CYCLIC GILTS TREATED WITH PORCINE LUTEINIZING HORMONE (pLH)

2.1 Introduction

Recent research has demonstrated that porcine luteinizing hormone (pLH, Lutropin-V, Bioniche Animal Health Canada Inc., Belleville, Ontario, Canada) can reliably synchronize ovulation in weaned sows. These studies, typically involving a synchronization protocol with a 600 IU equine chorionic gonadotropin (eCG) treatment on the day of weaning and a 5 mg pLH treatment 72 to 80 hours later, report animals ovulating between 34.25 to 42.5 h (Cassar *et al.* 2005) and 30 to 42 h (Candini *et al.* 2001) following treatment. Average treatment to ovulation intervals of 35.7 h (Candini *et al.* 2001), 38.2 h (Cassar *et al.* 2005), and 39.2 h (Viana *et al.* 2002) have been described. To date, little research has been conducted on the use of pLH in gilts. Furthermore, the work that has been published involved the use of this hormone for the induction of puberty (Gama *et al.* 2005). Given that pLH has not been fully evaluated for application in the cyclic gilt, there is a need to establish if this product is appropriate for induction of ovulation in these animals.

The importance of endogenous LH release during the follicular phase has been well established. In particular, developing follicles appear to become dependant on LH for their continued growth (Guthrie 2005), and the LH surge initiates ovulation (Elsasser 1982). Evaluating the endocrine response to administered exogenous pLH would, therefore, be useful in understanding how this treatment can synchronize ovulation. Furthermore, insight into the animals' response to exogenous pLH will also help to determine if the timing of the pLH treatment is appropriate to elicit the desired response. Unfortunately, all previous research in regards to the action of pLH has focused on dose-response studies to determine the dosage required to reliably synchronize ovulation (Viana *et al.* 2005; Bennett-Steward *et al.* 2007), without establishing the physiological responses associated with effective treatment.

Various studies have reported the influence of exogenous hormone treatment and control of the estrous cycle on embryo quality; one example was the hormonal treatment of gilts which resulted in the production of greater percentages of degenerated embryos (Ziecik *et al.* 2005). In light of the recent advancements made with fixed-time artificial insemination (AI) and embryo transfer technologies in swine (Niemann and Rath 2001), this can be a relevant issue. In pigs, stimulation protocols for these technologies typically involve the use of equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG) to synchronize ovulation (Estill 1999). However, these hormones may detrimentally influence oocyte maturation (Wiesak *et al.* 1990) and embryo quality in terms of viability (Ziecik *et al.* 2005; do Lago *et al.* 2005) and total cell numbers (Hazeleger *et al.* 2000). These problems may also arise with exogenous pLH treatment. As part of embryo evaluation studies, differential staining procedures have been established for both bovine and mouse embryos, allowing for differentiation between trophectoderm (TE) and inner cell mass (ICM) cells of embryos (Thouas *et al.* 2001; Fouladi-Nashta *et al.* 2005). Other studies have also used this procedure on porcine embryos (Kim *et al.* 2004, 2005). Establishing this as a routine method for swine would be useful to evaluate porcine embryo quality following hormonal stimulation.

The primary objective of this study was to determine if pLH can reliably synchronize ovulation in cyclic gilts and establish if the timing of ovulation is similar to that seen in weaned sows. The endocrine response of the gilts to pLH treatment was also evaluated through intensive blood sampling and hormone analysis to establish the timing of the endogenous LH surge in relation to pLH treatment. Additionally, differential staining procedures were tested on embryos recovered from these animals to determine proper stain concentrations and incubation timing for porcine embryos, in order to establish a reliable staining technique.

2.2 Materials and Methods

2.2.1 Animals

This study was conducted in accordance with Canadian Council on Animal Care guidelines and was approved by the University of Alberta Faculty Animal Policy Welfare

Committee (Protocol DYCK-2006-40). Four littermate pairs of gilts (Hypor F1, Landrace x Large White) that had completed their first estrous cycle were used for this study. Gilts were selected based on their weight (difference of 15 kg or less) and the synchrony of their first estrus. Animals were split into two groups and allowed to complete one (group 1) or two (group 2) more reproductive cycles. Animals were housed in individual crates and fed at 1.5 x maintenance, with maintenance adjusted for growth calculated as 112 x Kcal ME kg^{-1} $BW^{0.75}$ (NRC 1998), where ME is metabolic energy and BW is body weight in kg. Animals were exposed to 12 hours light and 12 hours dark each day. Subsequent estrus was detected once per day using fence-line contact with two mature boars, with a minimum five minutes of contact per boar.

2.2.2 Altrenogest Treatment

The treatment schedule is outlined in Figure 2.1. Starting on day 5 to 16 of their estrous cycle, animals were given oral altrenogest treatment (Regu-mate™, 15 mg per day, Intervet Canada Inc., Whitby, Ontario, Canada) for 14 days to synchronize their subsequent estrus and to allow the animals to fit into the hormonal stimulation protocol. Upon withdrawal of altrenogest, animals were checked for estrus twice a day (approximately 8:00 AM and 4:00 PM), as described earlier. Upon detection of standing estrus (day 0 of the estrous cycle), real time transcutaneous ultrasonography (Pie medial Scanner 200, model 4[illegible]80, Maastricht, the Netherlands, using a 5.0/7.5 MHz probe, model 401502, Maastricht, the Netherlands), conducted at the same frequency as estrus detection, was used to record ovarian follicular development and determine the timing of ovulation.

2.2.3 Surgery

On day 10 of the estrous cycle following altrenogest treatment, animals underwent surgery for the insertion of an indwelling jugular catheter (Tygon tubing, S-54-HL, lot # 183840, ID 0.050 inches, OD 0.090 inches, WALL 0.020 inches, Saint-Gobain Performance Plastics, Akron, OH, USA) via the cephalic vein (Cosgrove *et al.* 1993). Following surgery, the animals' feed and water intake, as well as rectal temperature, was measured three times daily for two days to confirm appropriate recovery. Catheters were flushed with a 10 IU heparin/ml heparin-saline solution (Heparin LEO, LEO Pharma Inc., Thornhill, Ontario, Canada) every twelve hours to keep the catheter patent. Animals were monitored daily following the recovery period for signs of infection.

2.2.4 Hormonal Stimulation Protocol

Animals within a littermate pair were randomly assigned to either control or pLH treatments following surgery. After a four-day recovery period (approximately day 14 of the estrous cycle), animals received two treatments of 500 µg cloprostenol (Estrumate, Schering-Plough Animal Health, Point-Claire, Quebec, Canada) intramuscularly (im), eight hours apart. Twenty four hours after the first cloprostenol treatment (day 15), animals received 600 IU eCG im (Folligon, Intervet Canada Inc., Whitby, Ontario, Canada). Then, 80 hours after the eCG treatment (day 18), animals received either 5 mg pLH im (Lutropin-V, Bioniche Animal Health Canada Inc., Belleville, Ontario, Canada) or saline, depending on their assigned treatment.

2.2.5 Blood Sampling

Blood samples (3 ml) were collected into heparinized tubes (1000 IU heparin/ml solution, two drops per tube) in conjunction with catheter flushing every twelve hours from the evening of the day of surgery until day 18 of the gilts' estrous cycle. Eight hours prior to pLH treatment on day 18, sampling frequency increased to every four hours until the animal was no longer exhibiting standing estrus. In a subset of animals (n = 4), blood samples were taken at 30-minute intervals for 4 hours following pLH/saline treatment. Samples were centrifuged for 15 minutes at 2500g, and the plasma was transferred into separate tubes and stored at approximately –30°C until analysis. Animals were monitored (rectal temperature, feed and water intake, visible signs of distress) over the period of blood sampling; however, problems with anemia did not occur. Catheters were locked with the heparin flush and an antibiotic (Cefazolin) at the conclusion of blood sampling.

2.2.6 Determination of Ovulation Timing and Breeding

To detect the onset of estrus and ovulation following hormonal stimulation, estrus detection and transcutaneous real-time ultrasonography was performed every eight hours (8:00 AM, 4:00 PM, 12:00 AM) beginning eight hours prior to pLH/saline treatment.

Estimated time of ovulation was determined using a method similar to that described by Almeida *et al.* (2001) adjusted for frequency of ultrasonography. The relationship between duration of estrus and timing of ovulation was determined by dividing the time between onset of estrus and ovulation by the total duration of estrus (% ovulation). Again, fence-line boar exposure was performed with two boars for 5 minutes minimum per boar. pLH-treated animals were inseminated 32 and 40 hours after the pLH treatment with pooled semen from Duroc boars from the University of Alberta Swine Research and Technology Centre designated for this experiment. The insemination doses of 50 ml, were extended in Beltsville thawing solution (Minitube of America, Verona, Wisconsin, USA), and contained 3 x 10^9 motile and morphologically normal spermatozoa. All inseminations were performed with semen that was less than three days old. Control animals were inseminated 16 hours following initial detection of standing estrus, and then every 24 hours standing estrus was observed with the same source and dose of semen, until the animal was no longer exhibiting estrus.

2.2.7 Embryo Collection

At day 6.4 (± 0.3) of gestation (day of ovulation was considered to be day 1), animals were euthanized on site. Reproductive tracts were recovered and ovulation rate was determined by counting the number of corpora lutea (CL) on each ovary. To ensure any hidden CLs were not missed, several incisions were made in the ovarian tissue.

The broad-ligament was cut away from the uterus starting at the cervix and moving towards the oviduct. An incision was made at the bottom of the uterine body near where it intersects with the cervix, and a modified 10 ml syringe cylinder with the top remolded was inserted into the incision and secured in place. A 25 ml volume of a pre-warmed (~35°C) phosphate buffered saline (PBS) containing 5 mg/ml of bovine serum albumin (BSA) was injected into each uterine horn via the oviduct at the utero-tubal junction. The PBS solution was then pushed through the uterus until it drained out through the modified syringe into a 50 ml plastic conical tube (Falcon tube, Becton Dickinson Labware, Franklin Lakes, New Jersey, USA). This procedure was performed twice per uterine horn. The flush solution was kept in a warmed, insulated box to maintain its temperature at ~35°C.

2.2.8 Embryo Classification and Staining

Embryos were identified under a dissecting microscope (Leica MZ 12_5, Leica Microsystems Ltd., Switzerland), harvested and classified by developmental stage at 25 to 50x magnification. All embryos from the non-expanded blastocyst stage to more advanced stages of development were exposed to a modified differential staining procedure utilizing the two fluorochromes propidium iodide and bisbenzimide to visualize TE and ICM cells (Thouas *et al.* 2001). Briefly, embryos were initially placed in 500 µl of 0.1 mg/ml propidium iodide (P4864, Sigma Aldrich Inc., St. Louis, Missouri) in PBS and 1% Triton-X (T9284, Sigma Aldrich Inc., St. Louis, Missouri) solution, for 45 to 60 seconds and then washed three times in 100% ethanol. Embryos were then transferred to 500 µl of 0.025 mg/ml bisbenzimide H 33258 trihydrochloride (B2883, Sigma Aldrich Inc., St. Louis, Missouri) in 100% ethanol and left to incubate overnight at 5°C. These parameters were based on the bovine protocol from Thouas *et al.* (2001), as their composition is similar to porcine embryos. However, because it was unclear if these parameters would be suitable, timing of incubation in the first stain was varied to see which timing produced the best staining results and if the length of incubation damaged embryo integrity. Embryos were then mounted in glycerol and examined under ultraviolet light using an inverted microscope (Olympus IX81-UCB-2, Tokyo, Japan) at 200x magnification to evaluate the staining. Preliminary cell counting was performed using Metamorph software (Version 7.1.0.0, Molecular Devices).

2.2.9 Luteinizing Hormone Assay

Determination of pLH concentrations was performed using 200 µl of plasma which was assayed in triplicate using the homologous double antibody radioimmunoassay (RIA) as described by Wellen (2005). A second assay, in which specific high potency samples were diluted 5- or 10-fold, was also performed. The intra-assay variance was 9.8% and 8.1% for the original and the rerun assays, respectively. The inter-assay variance was 13.6%. The average sensitivity across the two assays, defined as 84% of total binding, was 0.10 ng/ml. Plasma LH concentrations are expressed as ng equivalents of the purified pLH preparation AFP12389A (A.F. Parlow, Harbor UCLA Medical Center, 1000 West Carson Street, Torrance California, USA 90509) with a stated potency of 2.2 X NIH S1.

To calculate the point at which the LH surge was considered to have commenced, a method similar to that described by Roski (2004) was used. Briefly, for each gilt, the average LH baseline was calculated using all samples taken at 12-hour intervals. The standard deviation of these samples was also calculated. The occurrence of consecutive samples that were two standard deviations above the LH baseline was considered to be the point at which the LH surge had begun. Once LH concentrations had declined below this level, the LH surge was deemed to be complete.

2.2.10 Statistical Analysis

All data were verified for normality and equality of variance using the PROC UNIVARIATE and PROC GLM (Bartlett and Levene tests) procedures in SAS (SAS Institute, Cary, NC, USA, 1990). Data that met the assumptions of typical statistical tests were analyzed using the PROC MIXED procedure. Any others were transformed to a normal distribution and then tested using the PROC MIXED procedure. All proportional data were arcsin transformed to normalize the data and then tested using the PROC MIXED procedure. The design was analyzed as a randomized complete block design with no replication within the blocks. The fixed effect in the model was considered to be the treatment (pLH or saline) and the random effect in the model was the pairs of littermates. The experimental unit was the gilt. Number of estrous cycles was analyzed as a covariate, but was not significant ($p > 0.05$). All results presented are the least square means and standard errors calculated by SAS following PROC MIXED analysis.

2.3 Results

2.3.1 Estrus and Ovulation Timing

As presented in Table 2.1, no differences in the timing and duration of estrus and timing of ovulation within the estrus period were found between control and pLH-treated animals. All animals were at similar weights at all the points of measurement, indicating metabolic status was not a factor.

The duration of standing estrus following the hormonal stimulation treatments was similar to control gilts, with an overall mean of 2.1 ± 0.2 days or 50.7 ± 3.7 hours. There was no difference in the timing of ovulation in relation to the duration of estrus (73.9 ± 7.1% for control animals and 70.3 ± 7.1% for the pLH group). The interval between pLH treatment and ovulation was on average 38.4 ± 3.7 hours (range of 36.0 to 44.0 h) following treatment. Ovulation tended to occur later in the control animals, taking place approximately 8 hours later when compared to the pLH-treated animals in relation to the timing of treatment (p = 0.06). Since the pLH group tended to ovulate sooner in relation to treatment, the insemination prior to ovulation corresponded more closely to ovulation in pLH-treated gilts (11.9 ± 2.2 h for control and 4.0 ± 2.2 h for the pLH group; p = 0.06). The variance in the timing of ovulation was equal for both groups.

2.3.2 LH Measurements

On average, the onset of the endogenous LH surge began before estrus was detected, and ovulation occurred 39.4 to 45.4 hours after the defined start of the natural or pLH-enhanced LH surge (Table 2.2). Ovulation occurred 33.4 ± 2.7 hours after the peak of the natural or pLH-enhanced LH surge in both groups. Interestingly, in most gilts, exogenous pLH was given before the start of the endogenous LH surge, which indicates that the treatment was applied at an appropriate time to marginally advance the surge if it has the capacity to do so. Parameters of the endogenous or pLH-enhanced LH surge are presented in Table 2.3. There was no difference in the duration of the LH surge among treatments. The maximum pLH concentration reached at the peak of the LH surge (Figure 2.2), as well as the amplitude of the LH surge (Figure 2.3) were higher in pLH-treated animals than in the control treatment (p = 0.01).

LH profiles for pLH and control animals are shown in Figures 2.4 to 2.11. These profiles confirm that the exogenous pLH does increase the overall plasma concentrations of LH, thereby increasing the peak and amplitude of the LH surge. Although the treatment of the pLH animals usually occurred before the start of the endogenous LH surge, it can be seen that the timing varied among gilts. In the case of the pLH-treated animal shown in Figure 2.7, pLH treatment was given after the endogenous LH surge was initiated which may influence the effectiveness of the pLH treatment, although this was not the case here.

In the subset of four animals in which blood samples were taken at a half hour frequency for four hours immediately following pLH or saline (Figures 2.12 to 2.15), there is clearly a response to pLH treatment, shown by the elevated circulating LH concentrations. However, the response may not be immediate in all cases, as LH concentration in one pLH-treated animal is similar to its' paired littermate control up until 3.5 hours after treatment, as shown in Figures 2.12 and 2.13. Regardless, there was a significant difference in the variation among treatments in the interval between treatment and the time at which the peak of the endogenous or pLH-enhanced LH surge was reached ($p = 0.01$), with the controls being more variable.

2.3.3. Embryo Data

As summarized in Table 2.4, all animals in the study were of similar metabolic state and day of gestation at the time of euthanasia. Although no significant differences ($p > 0.05$) were noted in any of the reproductive parameters measured after euthanasia (Table 2.4), all measures of ovarian development and embryo numbers were similar among control and pLH-treated gilts.

There was no difference among treatment groups in non-fertilized, degenerated, very early stage, or advanced stage embryos collected (Table 2.5). There appears to be some heterogeneity of embryonic development within litters, with a range of developmental stages being seen in both treatments (Figure 2.16). On the other hand, three individual gilts (two control animals and one pLH-treated animal) showed homogenous litters made up mainly of expanded blastocysts.

With regards to the staining results, the embryos from all animals were used to test different concentrations of stain and detergent, as well as the incubation timing of the first stain. Therefore, comparative results cannot be drawn from these embryos. However, for future staining, incubation time in the first stain should be reduced to limit the disruption of and penetration of the stain into the inner cell mass, as all blastomeres of the embryos were fully stained with both fluorochromes (Figure 2.17). A total of 103 embryos were stained.

2.4 Discussion

The results of the present study established that a 5 mg dose of pLH is effective in stimulating ovulation in cyclic gilts and that the time of ovulation following treatment falls within the ranges found for weaned sows (Candini *et al.* 2001; Viana *et al.* 2002; Cassar *et al.* 2005). As ovulation occurred later in the control animals, taking place approximately 8 hours later when compared to the pLH-treated animals in relation to the timing of treatment, it would appear that pLH-treated animals ovulated more promptly and in a more synchronized manner than control animals. As a consequence, the timing of the first fixed-time insemination appears to be appropriate for the pLH-treated group. However, it was observed that the second insemination, regardless of treatment, was most often redundant and occurred after ovulation. As a result, it is proposed that in subsequent studies in which ovulation is being observed, AI should cease once ovulation is confirmed rather than depending on the duration of estrus. Given that real-time ultrasound detection of follicular development and ovulation is not practical in production settings, it is important to determine reliable ovulation timing following pLH treatment in further studies involving cyclic gilts. This will indicate the feasibility of applying a single fixed-time insemination in gilts when ovulation is induced with pLH. Unfortunately, due to the small sample size used for this pilot study, these trends in reproductive response to pLH were not statistically significant. However, the physiological information obtained provides a sound foundation for subsequent studies to evaluate the response of a large number of gilts to pLH treatment.

The endocrine results indicate that the pLH treatment elicits a significant response in the gilts, although the response is prone to individual variation. Evaluating individual profiles in which the endogenous LH surge had not initiated at time of treatment, it appeared that the rate at which the peak of the LH surge was reached was influenced by pLH treatment. This will help to establish the mechanism by which pLH acts to influence ovulation. It has been suggested that follicles only require a certain threshold of LH stimulation to initiate the ovulatory process, and that the peak of the LH surge reached may not be as important, although timing of ovulation is closely related to the timing of peak LH (Soede *et al.* 1994). Therefore, if pLH treatment can cause this threshold to be reached more rapidly, it may in fact initiate a more synchronized ovulation. Our results indicate that pLH treatment enhances the endogenous LH surge, tending to result in the peak level of the surge to be reached more rapidly than in control animals. This finding is further supported by the finding that the variation in the interval between treatment and peak LH concentrations was significantly less in the pLH-treated gilts. Therefore, whether it is a threshold LH concentration or the peak of the surge that is the important factor to stimulate ovulation became irrelevant, as both occur sooner following pLH treatment. As well, a consistent relationship between the timing of the LH surge peak and ovulation was established regardless of treatment, indicating that once a threshold is reached, ovulation occurs within a consistent time period. Having observed the relationship between peak LH concentration and timing of ovulation and the fact that pLH treatment reduces the time it takes to reach this peak and with less variation, one would conclude that ovulation is stimulated sooner and more predictably by pLH. On the other hand, if the duration of the LH surge is altered, it may allow for further development of less developed follicles, and if the LH threshold is maintained for enough time, there could be ovulation of these less developed follicles later into the LH surge and after the initial round of ovulation. For example, it has been suggested that Meishan animals exhibit more uniform embryonic development, and that this is due to a shorter LH surge (Faillace and Hunter 1994). This may account for the observations by Pope *et al.* (1988) which found that the majority of follicles ovulate simultaneously, but a minority of follicles ovulates over a longer period. This could explain the type of embryos recovered from one pLH-treated animal (pair 4) which had a range of development from degenerated embryos to hatched blastocysts. This animal received the pLH treatment prior to the LH surge (Figure 2.11) and had a rapid endocrine response (Figure 2.15) but had a similar duration of the LH surge when compared to other pLH-treated gilts, so it could be possible that this animal had a longer duration of ovulation due to the rapid response although the overall duration of the surge was similar to the other animals. Despite apparent differences in the developmental stages of the embryos obtained from individual animals treated with pLH, the LH surge characteristics do not appear to differ between them (Figures 2.5, 2.7, 2.9, and 2.11). This would indicate the pLH ovulation threshold value may vary among individuals or that the inherent maturational state of follicles induced to ovulate is a key factor in determining subsequent developmental competence of the embryo. With regard to the one pLH-treated animal that received treatment following the onset of the LH surge, treatment to ovulation interval was not shorter than other pLH-treated gilts, the preovulatory follicle size was not reduced, and well-developed embryos were obtained; therefore, the small delay in pLH treatment in relation to the onset of the endogenous LH surge does not appear to reduce the ability of pLH to synchronize ovulation in this case.

Few conclusions can be made based on the embryo data obtained due to the lack of statistical significance. It should be noted that one control animal was euthanized earlier than the others (day 4.8), possibly skewing the results. This was indicated by the inclusion of day of gestation when evaluating the total number of blastocysts recovered. In this case, the day of gestation was a significant covariate ($p = 0.01$). Some morula stage embryos from this animal were allowed to culture overnight before staining, and they were able to reach the blastocyst stage. Conceivably then, if this animal had been euthanized at a similar time to the others, these embryos would have been at the blastocyst stage. This illustrates the importance of the timing of embryo collection in regards to the stage of embryos obtained, particularly if they are to be used for embryo transfer procedures. It is clearly necessary to establish the time of collection in relation to time of ovulation to ensure embryos are collected at the proper embryonic stage.

A differential staining method first developed for bovine and mouse embryos (Thouas *et al.* 2001; Fouladi-Nashta *et al.* 2005) and applied in this study, appears to be applicable to porcine embryos. There was, however, over-staining by propidium iodide which resulted in all cells being stained and consequently we were unable to differentiate between the inner cell mass and trophectoderm cells. An adjustment in incubation time in the first stain may need to be made and most likely it should be less than 45 seconds but more than 10 seconds. This would be expected to decrease the disruption of cell integrity by the detergent and prevent the stain from penetrating the inner cell mass, but still allow for some staining of the outer cells. Since the intensity of the fluorescence seen was appropriate for cell counting, no change needs to be made in the stain concentration.

Overall, pLH treatment is appropriate for use in cyclic gilts and enhances the endogenous LH surge, resulting in more predictable timing of ovulation. A reliable hormonal stimulation protocol was established, providing the basis for future studies involving the use of pLH in cyclic gilts. The differential staining technique utilized in this study has potential; however, adjustments need to be made to the procedure to allow for differential staining.

2.5 References

Almeida, F.R.C.L., Mao, J., Novak, S., Cosgrove, J.R., Foxcroft, G.R. 2001. Effects of different patterns of feed restriction and insulin treatment during the luteal phase on reproductive, metabolic, and endocrine parameters in cyclic gilts. *J. Anim. Sci.* 79:200–212.

Bennett-Steward, K., Cassar, G., Plante, C., Friendship, R.M., Zak, L. 2007. Ovulation induction protocol using equine chorionic gonadotropin and porcine luteinizing hormone in weaned sows. *J. Swine Health Prod.* 15: 194-197.

Candini, P.H., Moretti, A.S., Silveria P.R.S., Zanella, E., Viana, C.H.C., Santos, I.C.C. 2001. Utilization of a protocol of synchronization of ovulation, induced by porcine LH, to perform insemination programs in fixed time, with one or two inseminations per sow. Sixth International Conference on Pig Reproduction, Columbia, MO. pp. 99.

Cassar, G., Kirkwood, R.N., Bennett-Steward, K., Friendship, R.M. 2005. Towards timed single insemination in sows. Proceedings of the 36th Annual Meeting of the American Association of Swine Veterinarians, Toronto, ON. pp. 353-356.

Cosgrove. J.R., Urbanski, H.F., Foxcroft, G.R. 1993. Maturational changes in gondotrophin secretion: the LH response to realimentation and a nocturnal increment in LH secretion of feed restricted gilts. *J. Reprod. Fert.* 98:293-300.

do Lago, V., Viana, W.L., Gama, R.D., de Campos Rosseto, A., Pinese, M.E., de Sant'Anna Moretti, A. 2005. Second oestrus synchronization and precocious embryo viability after puberty induction in gilts by the use of gonadotrophin treatment. *Reprod. Dom. Anim.* 40:141–144.

Elsaesser, F. 1982. Endocrine control of sexual maturation in the female pig and sexual differentiation of the stimulatory oestrogen feedback mechanism. In Control of Pig Reproduction. Cole, D.J.A, Foxcroft, G.R. Eds. pp. 93-116.

Estill, C.T. 1999. Current concepts in estrus synchronization in swine. Proceedings of the American Society of Animal Science. Southern Section ASAS Meeting, Memphis, TN. E38.

Faillace, L.S., Hunter, M.G. 1994. Follicle development and oocyte maturation during the immediate preovulatory period in Meishan and white hybrid gilts. *J. Reprod. Fert.* 101:571-76.

Fouladi-Nashta, A.A., Alberio, R., Kafi, M., Nicholas, B., Campbell, K.H.S., Webb, R. 2005. Differential staining combined with TUNEL labeling to detect apoptosis in preimplantation bovine embryos. Reproductive BioMedicine Online. 10:497-502.

Gama, R.D., Vianna, W.L., Pinese, M.E., de Campos Rosseto, A., de Sant'Anna Moretti, A. 2005. Different doses of porcine luteinizing hormone in precocious puberty induction in gilts. *Reprod. Dom. Anim.* 40:433-435.

Guthrie, H.D. 2005. The follicular phase in pigs: Follicle populations, circulating hormones, follicle factors, and oocytes. *J. Anim. Sci.* 83(Suppl.):E79-89.

Hazeleger, W., Bouwman, E.G., Noordhuizen, J.P.T.M., Kemp, B. 2000. Effect of superovulation induction on embryonic development on day 5 and subsequent development and survival after nonsurgical embryo transfer in pigs. *Theriogenology.* 53:1063-1070.

Kim, H-s., Lee, G-s., Hyun, S-h., Lee, S-h., Nam, D-h., Jeong, Y-w., Kim, S., Kang, S-k., Lee, B-c., Hwang, W-s. 2004. Improved in vitro development of porcine embryos with different energy substrates and serum. *Theriogenology.* 61:1381-1393.

Kim, H-s., Lee, G-s., Hyun, S-h., Nam, D-h., Lee, S-h., Jeong, Y-W., Kim, S., Kim, J-h., Kang, S-k., Lee, B-c., Hwang, W-s. 2005. Embryotropic effect of glycosaminoglycans and receptors in development of porcine pre-implantation embryos. *Theriogenology.* 63:1167-1180.

Niemann, H., Rath, D. 2001. Progress in reproductive biotechnology in swine. *Theriogenology.* 56:12-91-1304.

NRC. 1998. *Nutritional Requirements of Swine.* Washington, DC, National Academy Press.

Pope, W.F., Wilde, M.H., Xie, S. 1988. Effect of electrocautery of nonovulated day 1 follicles on subsequent morphological variation among day 11 porcine embryos. *Biol. Reprod.* 39:882-887.

Roski, K. 2004. Ovulatory and reproductive characteristics of sows treated with an intravaginal GnRH agonist gel. Master's Thesis, North Carolina State University.

Soede, N.M., Helmond, F.A., Kemp, B. 1994. Periovulatory profiles of oestradiol, LH and progesterone in relation to oestrus and embryo mortality in multiparous sows using transrectal ultrasonography to detect ovulation. *J. Reprod. Fert.* 101:633-41.

Thouas, G., Korfiatis, N.A., French, A.J., Jones, G.M., Trounson, A.O. 2001. Simplified technique for differential staining of inner cell mass and trophectoderm cells of mouse and bovine blastocysts. Reproductive BioMedicine Online. 3:25-29.

Viana, C.H.C., Candini, P.H., Gama, R.D., Carbone, A., Santos, I.C.C., Vianna, W.L., Barnabe, R.C. 2002. Evaluation of a synchronization of ovulation protocol in cyclic sows. Proceedings of the International Pig Veterinary Congress, Ames, Iowa #678.

Viana, C.H.C., Castricini, E.S.C., Santos, I.C.C. 2005. Determination of the minimum porcine LH dose on synchronization of ovulation in sows. Presented at the International Congress on Pig Production, Amsterdam.

Wellen A. 2005. Reproductive performance and luteinizing hormone (LH) secretion in split-weaned sows. MSc Thesis, Wageningen Univ. Res., the Netherlands.

Wiesak, T., Hunter, M.G., Foxcroft, G.R. 1990. Differences in follicular morphology, steroidogenesis and oocyte maturation in naturally cyclic and PMSG/hCG-treated prepubertal gilts. *J. Reprod. Fert.* 89:633-41.

Ziecik, A.J., Biallowicz, M., Kaczmarek, M., Demianowicz, W., Rioperez, J., Wasielak, M., Bogacki, M. 2005. Influence of estrus synchronization of prepubertal gilts on embryo quality. *J. Reprod. Dev.* 51:379-384.

Table 2.1: Estrus characteristics and timing of ovulation for the peri-estrous periods before and after pLH or saline treatment (Least square means ± SEM)

	CONTROL (n = 4)	**pLH (n = 4)**	**p-value**
Duration of estrus after altrenogest treatment (h)	45.1 ± 4.8	54.2 ± 4.8	0.21
Onset of estrus to ovulation (h) after altrenogest treatment	36.0 ± 3.5	36.1 ± 3.5	0.99
% Ovulation* after altrenogest treatment	81.1 ± 6.1	66.5 ± 6.1	0.35
Weight at estrus after altrenogest treatment	146.4 ± 4.2	148.0 ± 4.2	0.80
Weight at surgery (kg)	151.5 ± 4.0	153.1 ± 4.0	0.79
Interval between estrus post-altrenogest and surgery (d)	8.5 ± 0.3	8.3 ± 0.3	0.64
Interval between estrus post-altrenogest and cloprostenol (d)	14.0 ± 0.2	13.8 ± 0.2	0.64
Interval between estrus post-altrenogest and eCG (d)	15.0 ± 0.2	14.8 ± 0.2	0.64
Interval between estrus post-altrenogest and pLH (d)	18.3 ± 0.2	18.2 ± 0.2	0.64
Estrus duration post-stimulation (h)	51.6 ± 5.6	49.9 ± 5.6	0.47
Interval - Onset of estrus to ovulation (h) post-stimulation	38.0 ± 3.9	34.2 ± 3.9	0.42
% Ovulation* post-stimulation	73.9 ± 7.1	70.3 ± 7.1	0.94
Interval - First insemination to ovulation (h)	**17.5 ± 2.8**	**6.0 ± 2.8**	**0.06**
Interval - Second insemination to ovulation (h)	-3.8 ± 3.3	-2.1 ± 2.9	0.72
Interval - Insemination closest to ovulation (h)	**11.9 ± 2.2**	**4.0 ± 2.2**	**0.06**
Interval - eCG to ovulation (h)	126.4 ± 3.7	118.4 ± 3.7	0.14
Interval – saline/pLH to ovulation (h)	**46.4 ± 3.7**	**38.4 ± 3.7**	**0.06**
Weight at onset of estrus post-stimulation (kg)	149.9 ± 4.2	152.5 ± 4.2	0.69

*% ovulation was calculated by dividing the time between onset of estrus and ovulation by the total duration of estrus

Table 2.2: The relationship between the LH surge and estrus timing, ovulation timing, and pLH or saline treatment (Least square means ± SEM)

	CONTROL (n = 4)	**pLH (n = 4)**	**p-value**
[1]Interval – Onset of LH surge to estrus (h)	-7.4 ± 3.9	-5.2 ± 3.9	0.47
[2]Interval – Onset of LH surge to ovulation (h)	-45.4 ± 2.3	-39.4 ± 2.3	0.10
[2]Interval - Peak of LH surge to ovulation (h)	-33.4 ± 2.7	-33.4 ± 2.7	1.00
[3]Interval – Treatment injection to onset of LH surge (h)	-1.0 ± 2.8	1.0 ± 2.8	0.88
[4]Interval – Treatment injection to peak of LH surge (h)	-13.0 ± 4.6	-5.0 ± 4.6	0.23

1- Interval is based on onset of estrus as time 0

2- Interval is based on the time of ovulation as 0

3- Interval is based on the onset of the LH surge as time 0

4- Interval is based on the peak of the LH surge as time 0

Table 2.3: LH surge characteristics (Least square means ± SEM); LH potencies expressed as ng equivalents of the purified pLH preparation AFP12389A with a stated potency of 2.2 X NIH S1.

	CONTROL (n = 4)	**pLH (n = 4)**	**p-value**
LH baseline (ng/ml)	0.5 ± 0.1	0.7 ± 0.1	0.16
Duration of LH surge (h)	29.0 ± 3.1	32.0 ± 3.1	0.57
Peak of LH surge (ng/ml)	**6.3 ± 1.7**	**11.8 ± 1.7**	**0.01**
Amplitude of LH surge (ng/ml)	**5.9 ± 1.2**	**11.0 ± 1.2**	**0.01**

Table 2.4: Gilt reproductive performance at day 6.4 (± 0.3) of gestation (Least square means ± SEM)

	CONTROL (n = 4)	**pLH (n = 4)**	**p-value**
Weight at euthanasia (kg)	155.1 ± 4.2	155.9 ± 4.2	0.91
Day of gestation	6.1 ± 0.2	6.6 ± 0.2	0.11
Ovulation rate	18.5 ± 3.2	23.5 ± 3.2	0.25
Ovarian cysts	0.8 ± 0.6	2.0 ± 0.6	0.24
Total embryos	15.3 ± 3.7	21.3 ± 3.7	0.21
% Embryo recovery	83.7 ± 6.5	88.4 ± 6.5	0.33

Table 2.5: Number of embryos, at various stages of development, recovered at day 6.4 (± 0.3) of gestation (Least square means ± SEM)

	CONTROL **(n = 4)**	**pLH** **(n = 4)**	**p-value**
Non-fertilized and degenerated	1.0 ± 2.2	3.8 ± 2.2	0.39
Early embryos (2-4 cell to morula)	3.0 ± 2.1	0.3 ± 2.1	0.20
Early blastocysts	2.0 ± 1.9	2.0 ± 1.9	1.00
Non-expanded blastocysts	2.8 ± 1.1	2.5 ± 1.1	0.88
Expanded blastocysts	6.3 ± 2.8	10.5 ± 2.8	0.27
De-expanded blastocysts	0.0 ± 0.4	0.5 ± 0.4	0.39
Hatched blastocysts	0.3 ± 1.0	1.8 ± 1.0	0.38
Total blastocysts	14.5 ± 5.1	14.0 ± 5.1	0.75

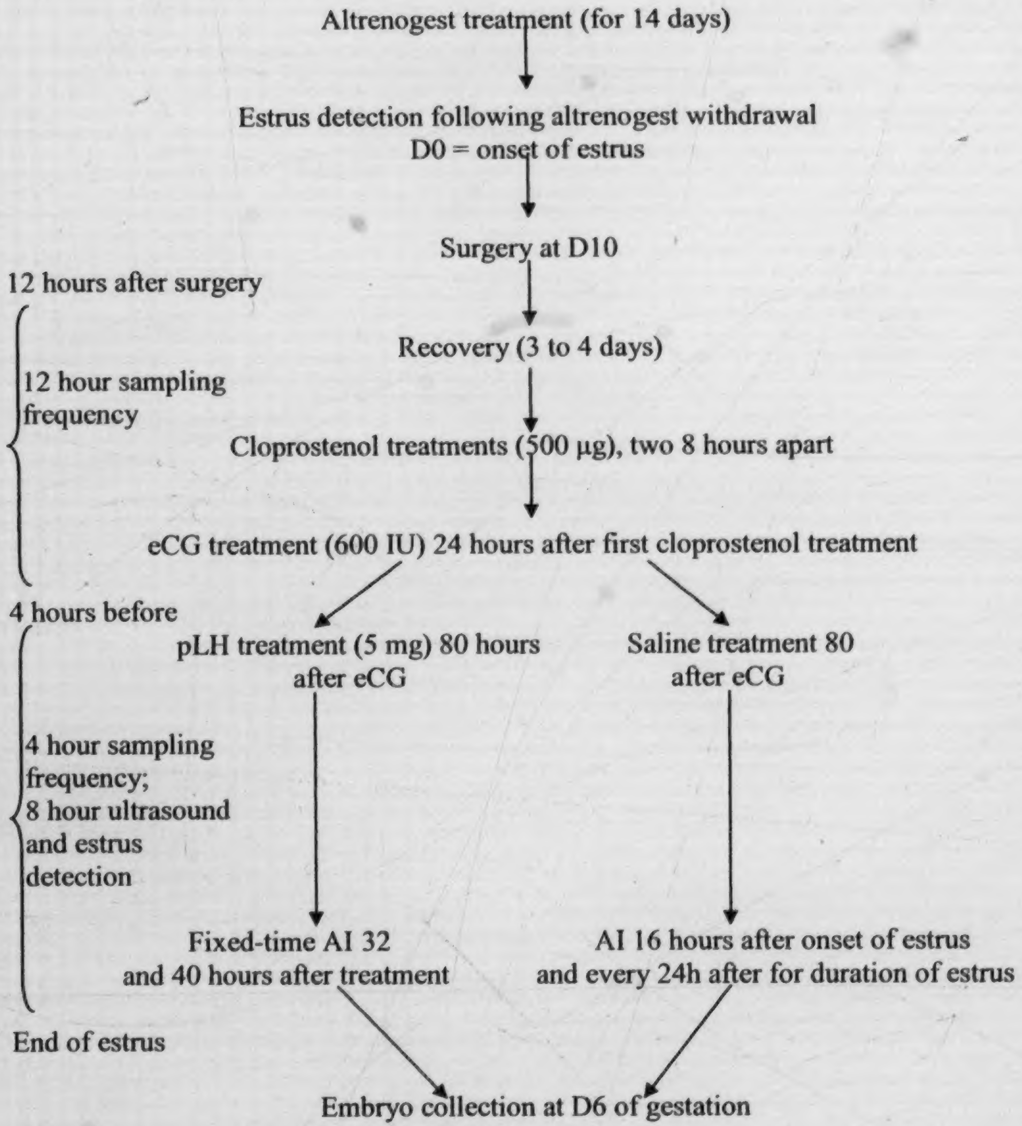
Altrenogest treatment (for 14 days)
Estrus detection following altrenogest withdrawal
D0 = onset of estrus
Surgery at D10
12 hours after surgery
12 hour sampling frequency
Recovery (3 to 4 days)
Cloprostenol treatments (500 µg), two 8 hours apart
eCG treatment (600 IU) 24 hours after first cloprostenol treatment
4 hours before
pLH treatment (5 mg) 80 hours after eCG
Saline treatment 80 after eCG
4 hour sampling frequency;
8 hour ultrasound and estrus detection
Fixed-time AI 32 and 40 hours after treatment
AI 16 hours after onset of estrus and every 24h after for duration of estrus
End of estrus
Embryo collection at D6 of gestation

Figure 2.1: Outline of the treatment schedule.

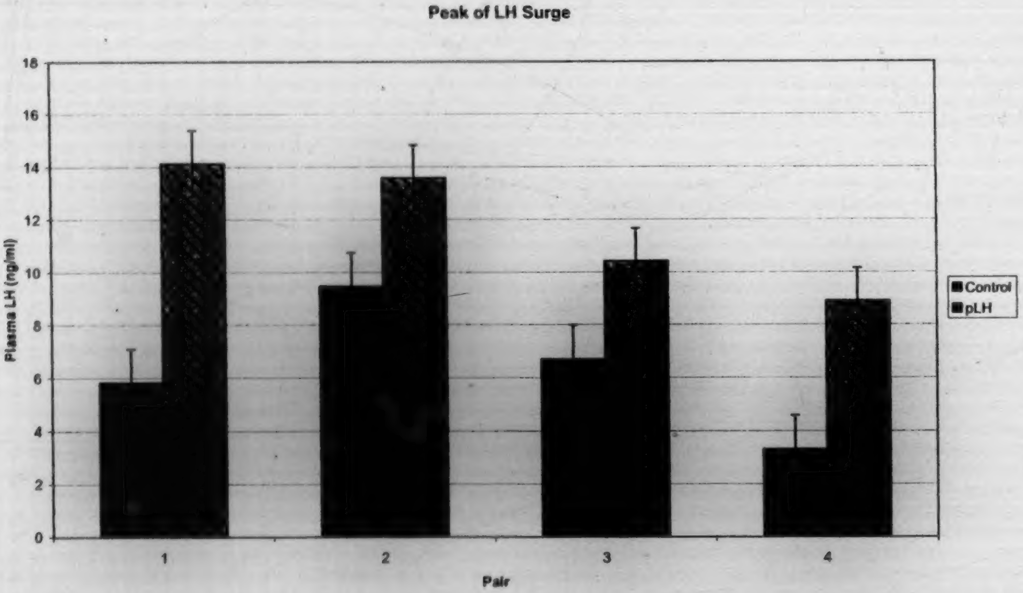

Figure 2.2: Peak plasma LH concentrations during the endogenous or pLH-enhanced LH surge for each gilt; plasma LH concentrations are expressed as ng equivalents of the purified pLH preparation AFP12389A with a stated potency of 2.2 X NIH S1.

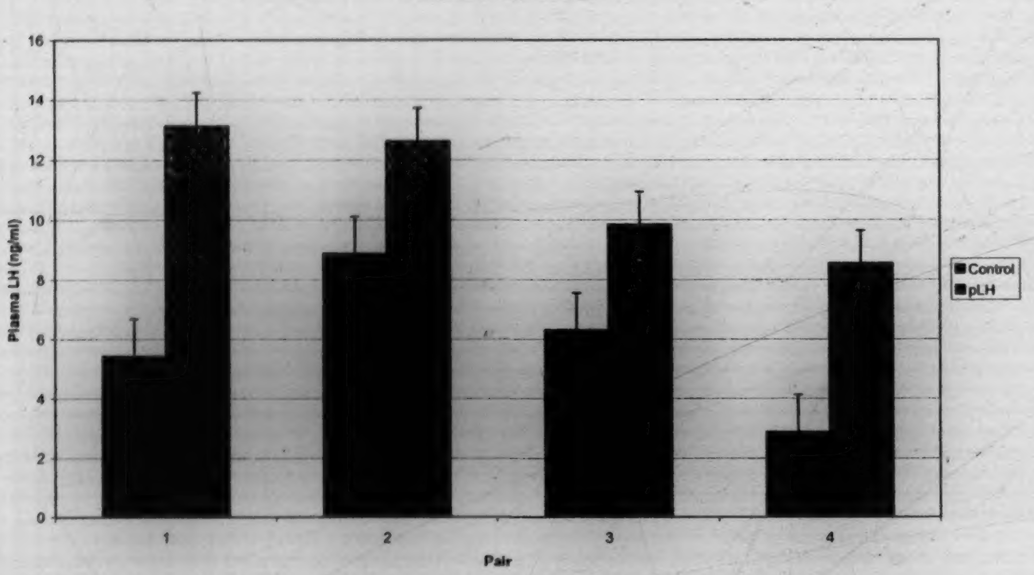

Figure 2.3: Amplitude of the endogenous or pLH-enhanced LH surge for each gilt; plasma LH concentrations are expressed as ng equivalents of the purified pLH preparation AFP12389A with a stated potency of 2.2 X NIH S1.

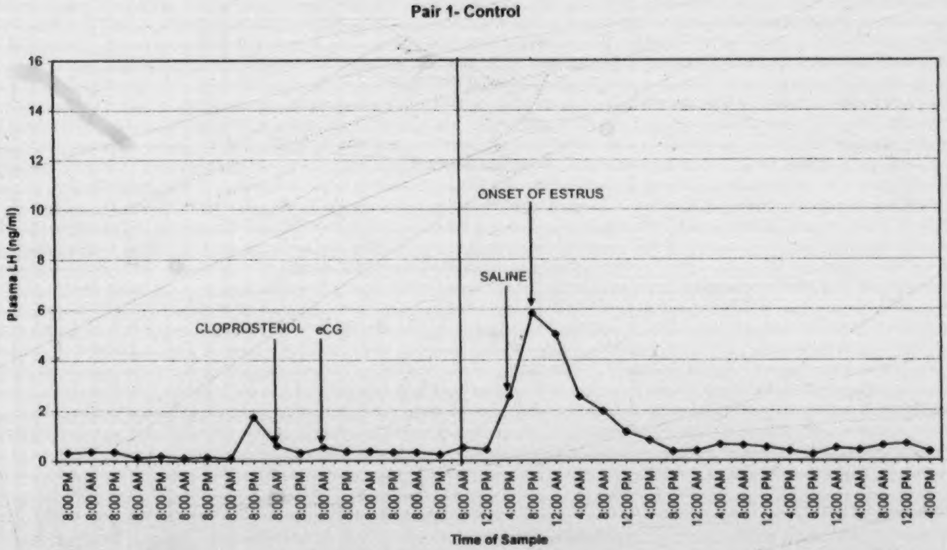

Figure 2.4: LH profile obtained for the control gilt in litter 1. Arrows indicate the timing of cloprostenol, eCG, and saline treatment and the onset of estrus. The graph is divided into 12-hour (left) and 4-hour (right) blood sampling frequencies. Plasma LH concentrations are expressed as ng equivalents of the purified pLH preparation AFP12389A with a stated potency of 2.2 X NIH S1.

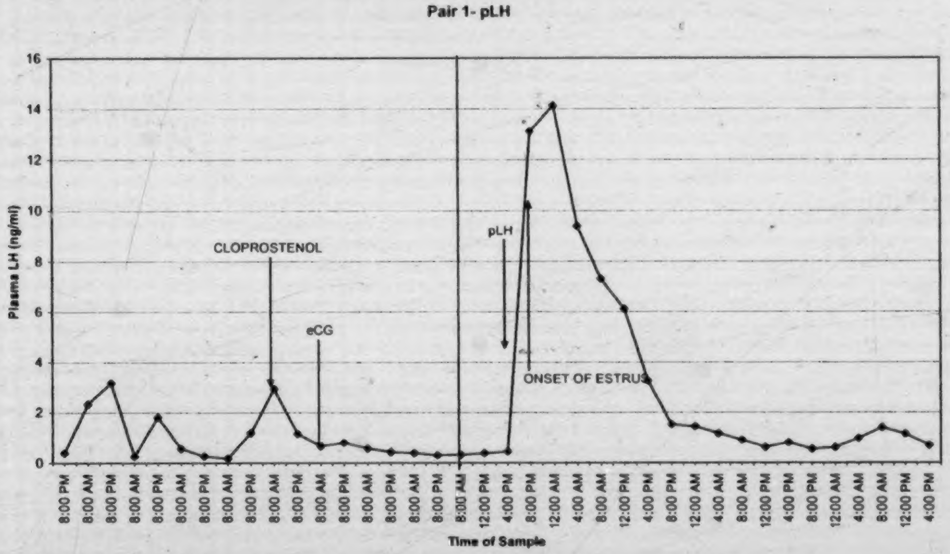

Figure 2.5: LH profile obtained for the pLH-treated gilt in litter 1. Arrows indicate the timing of cloprostenol, eCG, and pLH treatment and the onset of estrus. The graph is divided into 12-hour (left) and 4-hour (right) blood sampling frequencies. Plasma LH concentrations are expressed as ng equivalents of the purified pLH preparation AFP12389A with a stated potency of 2.2 X NIH S1.

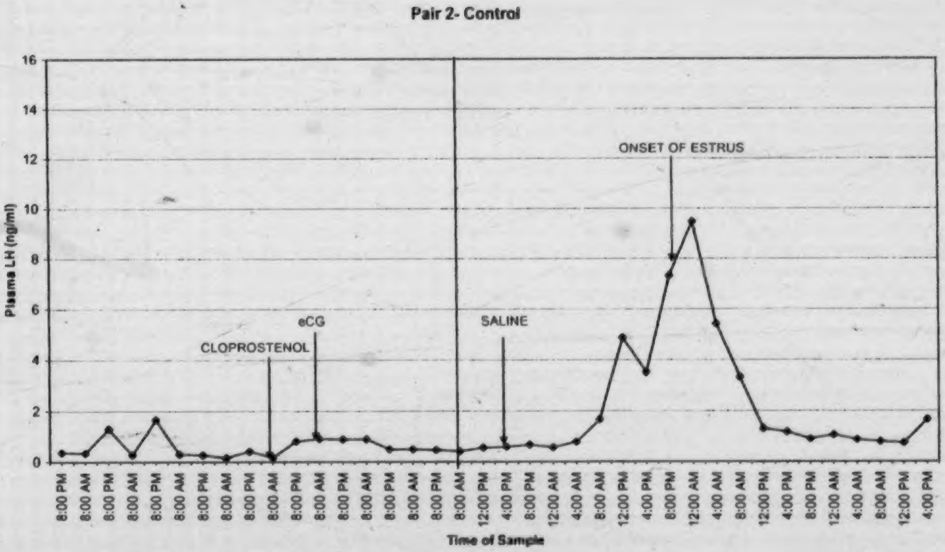

Figure 2.6: LH profile obtained for the control gilt in litter 2. Arrows indicate the timing of cloprostenol, eCG, and saline treatment and the onset of estrus. The graph is divided into 12-hour (left) and 4-hour (right) blood sampling frequencies. Plasma LH concentrations are expressed as ng equivalents of the purified pLH preparation AFP12389A with a stated potency of 2.2 X NIH S1.

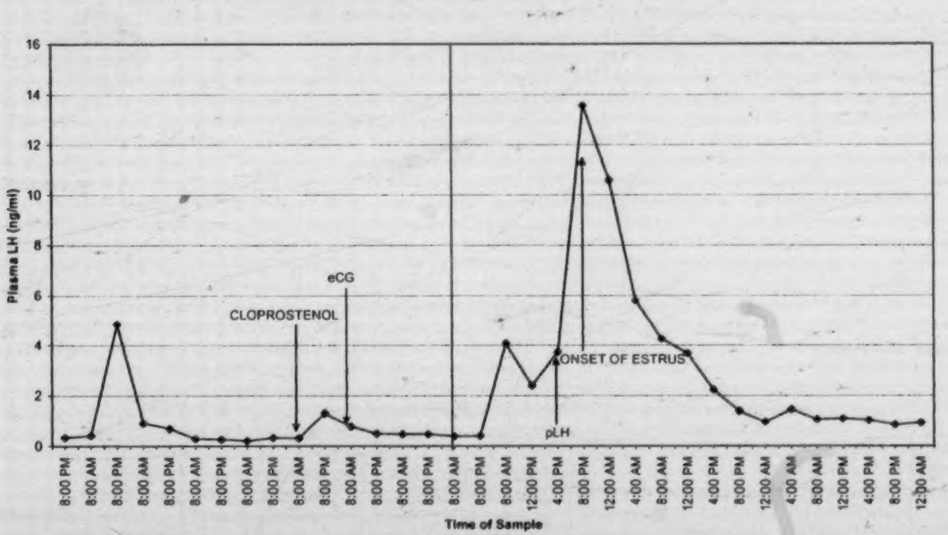

Figure 2.7: LH profile obtained for the pLH-treated gilt in litter 2. Arrows indicate the timing of cloprostenol, eCG, and pLH treatment and the onset of estrus. The graph is divided into 12-hour (left) and 4-hour (right) blood sampling frequencies. Plasma LH concentrations are expressed as ng equivalents of the purified pLH preparation AFP12389A with a stated potency of 2.2 X NIH S1.

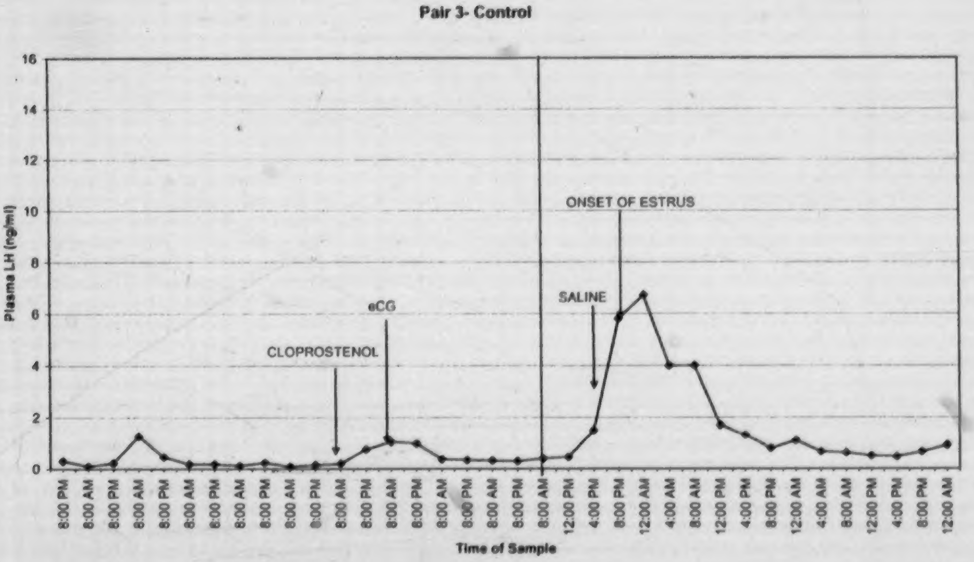

Figure 2.8: LH profile obtained for the control gilt in litter 3. Arrows indicate the timing of cloprostenol, eCG, and saline treatment and the onset of estrus. The graph is divided into 12-hour (left) and 4-hour (right) blood sampling frequencies. Plasma LH concentrations are expressed as ng equivalents of the purified pLH preparation AFP12389A with a stated potency of 2.2 X NIH S1.

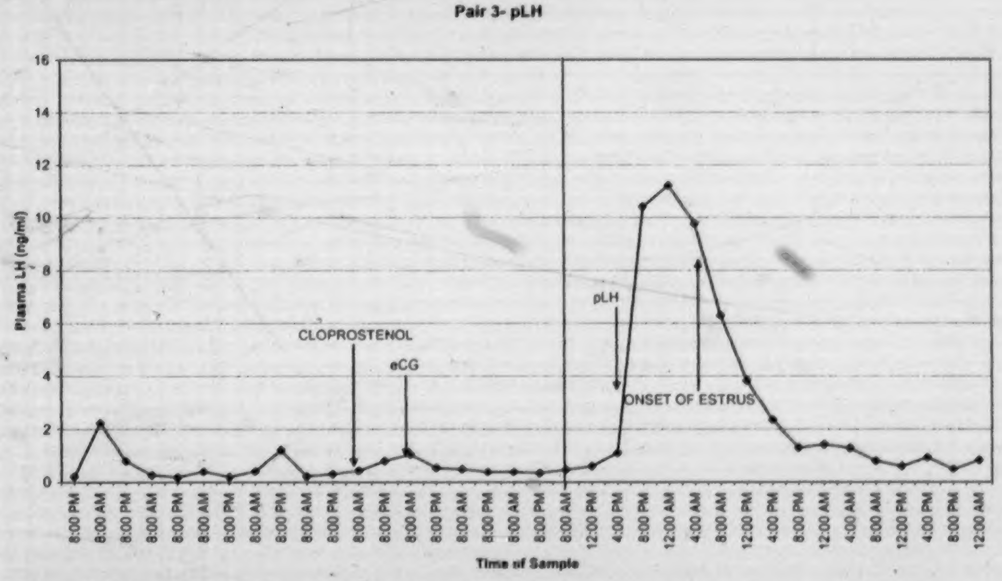

Figure 2.9: LH profile obtained for the pLH-treated gilt in litter 3. Arrows indicate the timing of cloprostenol, eCG, and pLH treatment and the onset of estrus. The graph is divided into 12-hour (left) and 4-hour (right) blood sampling frequencies. Plasma LH concentrations are expressed as ng equivalents of the purified pLH preparation AFP12389A with a stated potency of 2.2 X NIH S1.

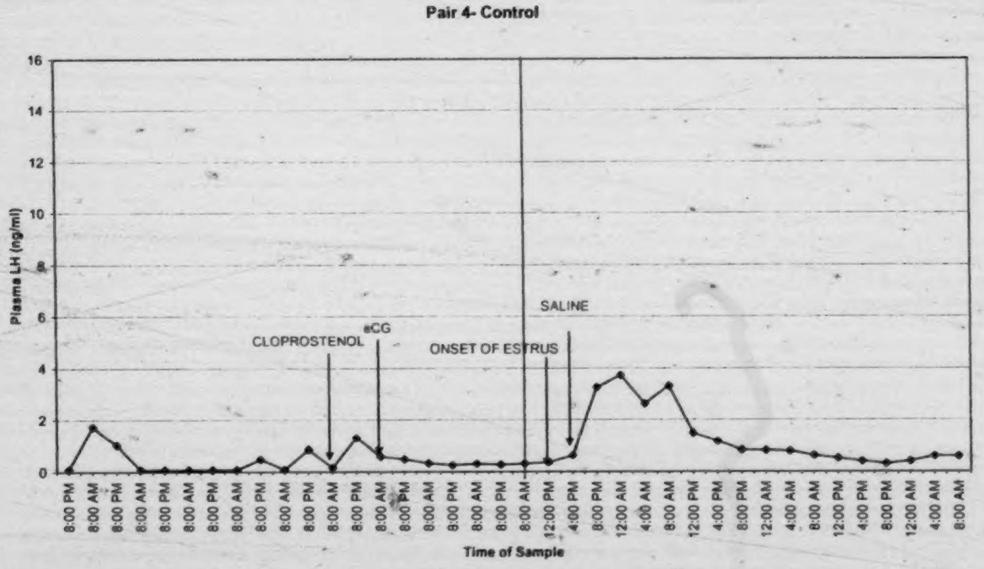

Figure 2.10: LH profile obtained for the control gilt in litter 4. Arrows indicate the timing of cloprostenol, eCG, and saline treatment and the onset of estrus. The graph is divided into 12-hour (left) and 4-hour (right) blood sampling frequencies. Plasma LH concentrations are expressed as ng equivalents of the purified pLH preparation AFP12389A with a stated potency of 2.2 X NIH S1.

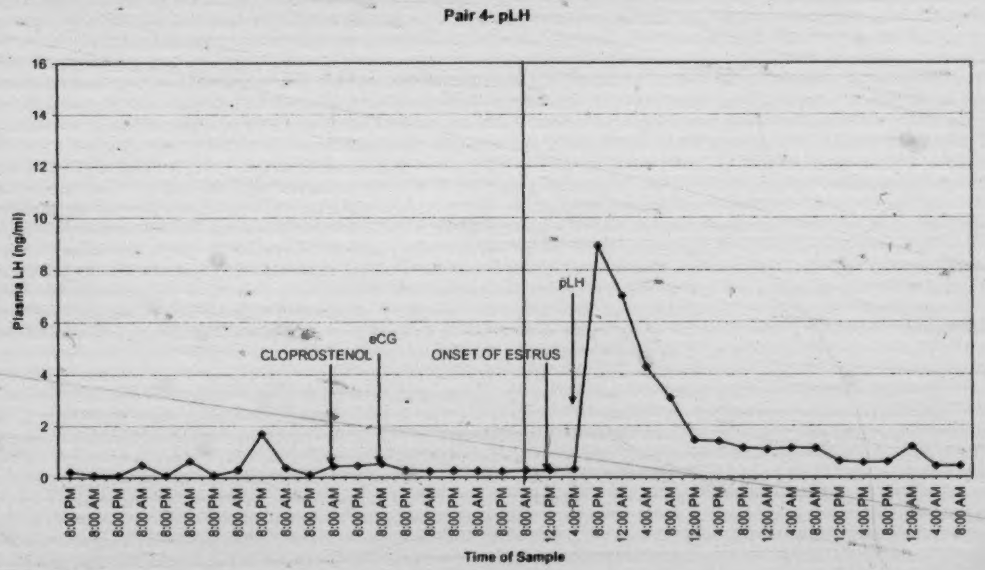

Figure 2.11: LH profile obtained for the pLH-treated gilt in litter 4. Arrows indicate the timing of cloprostenol, eCG, and pLH treatment and the onset of estrus. The graph is divided into 12-hour (left) and 4-hour (right) blood sampling frequencies. Plasma LH concentrations are expressed as ng equivalents of the purified pLH preparation AFP12389A with a stated potency of 2.2 X NIH S1.

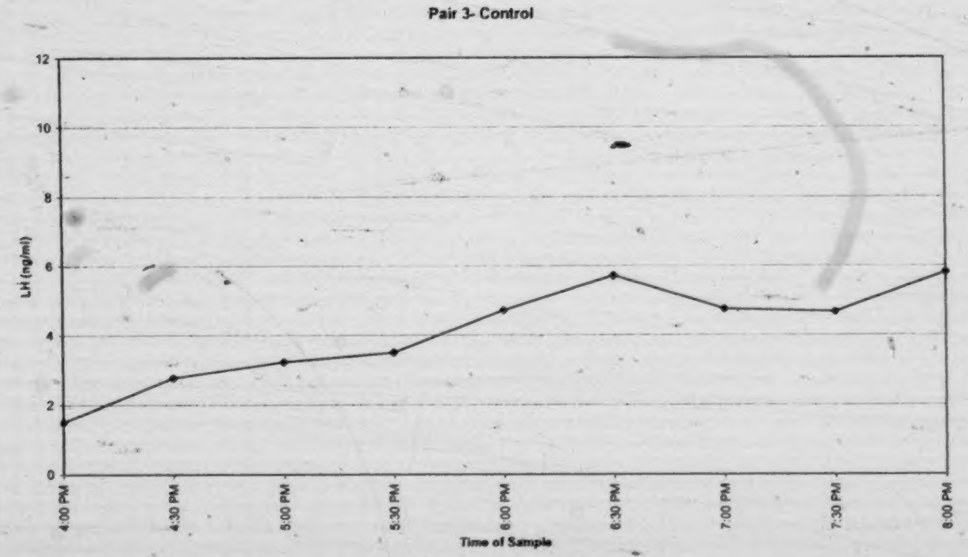

Figure 2.12: LH profile obtained for the control gilt in litter 3 immediately following saline treatment (at 4:00 PM). Samples were taken at 30 minute intervals for four hours. Plasma LH concentrations are expressed as ng equivalents of the purified pLH preparation AFP12389A with a stated potency of 2.2 X NIH S1.

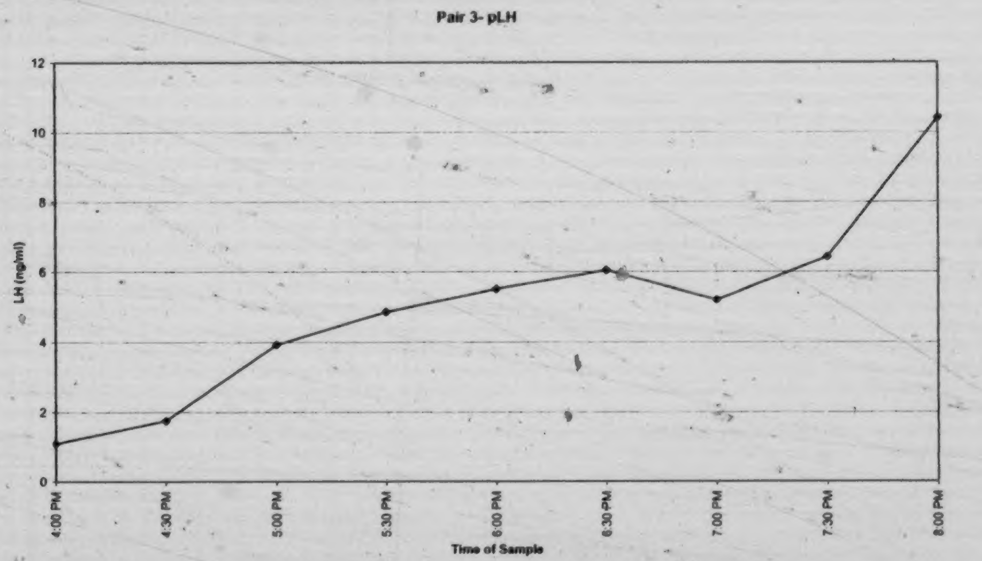

Figure 2.13: LH profile obtained for the pLH-treated gilt in litter 3 immediately following pLH treatment (at 4:00 PM). Samples were taken at 30 minute intervals for four hours. Plasma LH concentrations are expressed as ng equivalents of the purified pLH preparation AFP12389A with a stated potency of 2.2 X NIH S1.

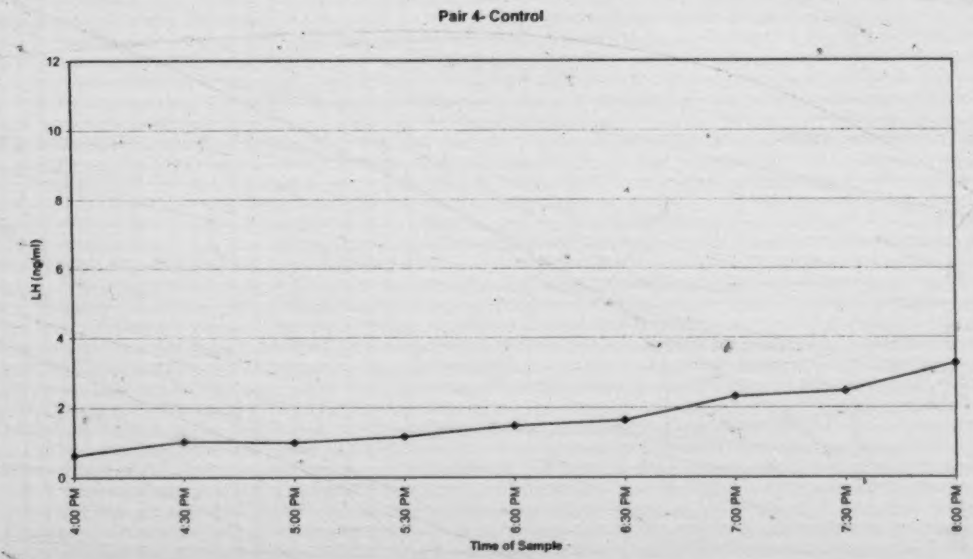

Figure 2.14: LH profile obtained for the control gilt in litter 4 immediately following saline treatment (at 4:00 PM). Samples were taken at 30 minute intervals for four hours. Plasma LH concentrations are expressed as ng equivalents of the purified pLH preparation AFP12389A with a stated potency of 2.2 X NIH S1.

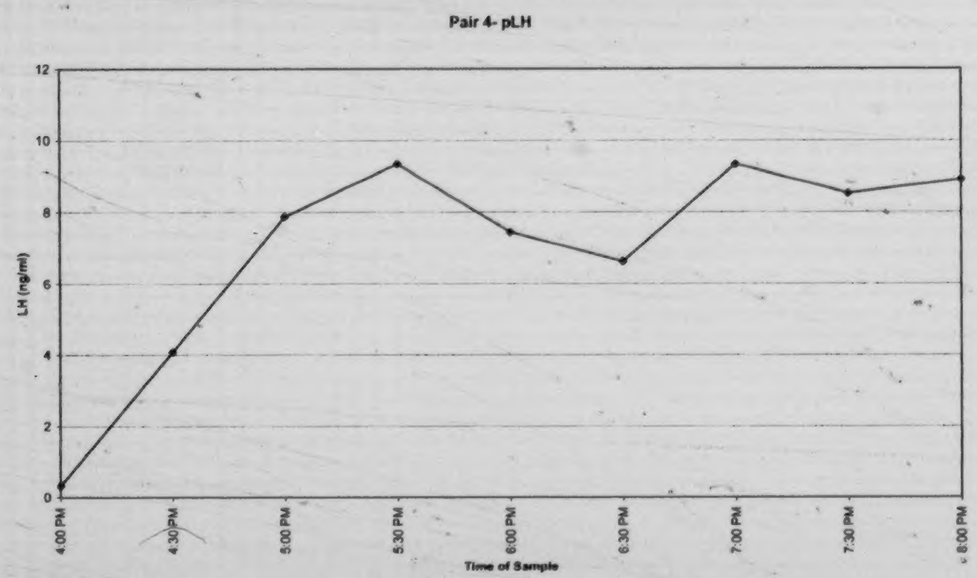

Figure 2.15: LH profile obtained for the pLH-treated gilt in litter 4 immediately following pLH treatment (at 4:00 PM). Samples were taken at 30 minute intervals for four hours. Plasma LH concentrations are expressed as ng equivalents of the purified pLH preparation AFP12389A with a stated potency of 2.2 X NIH S1.

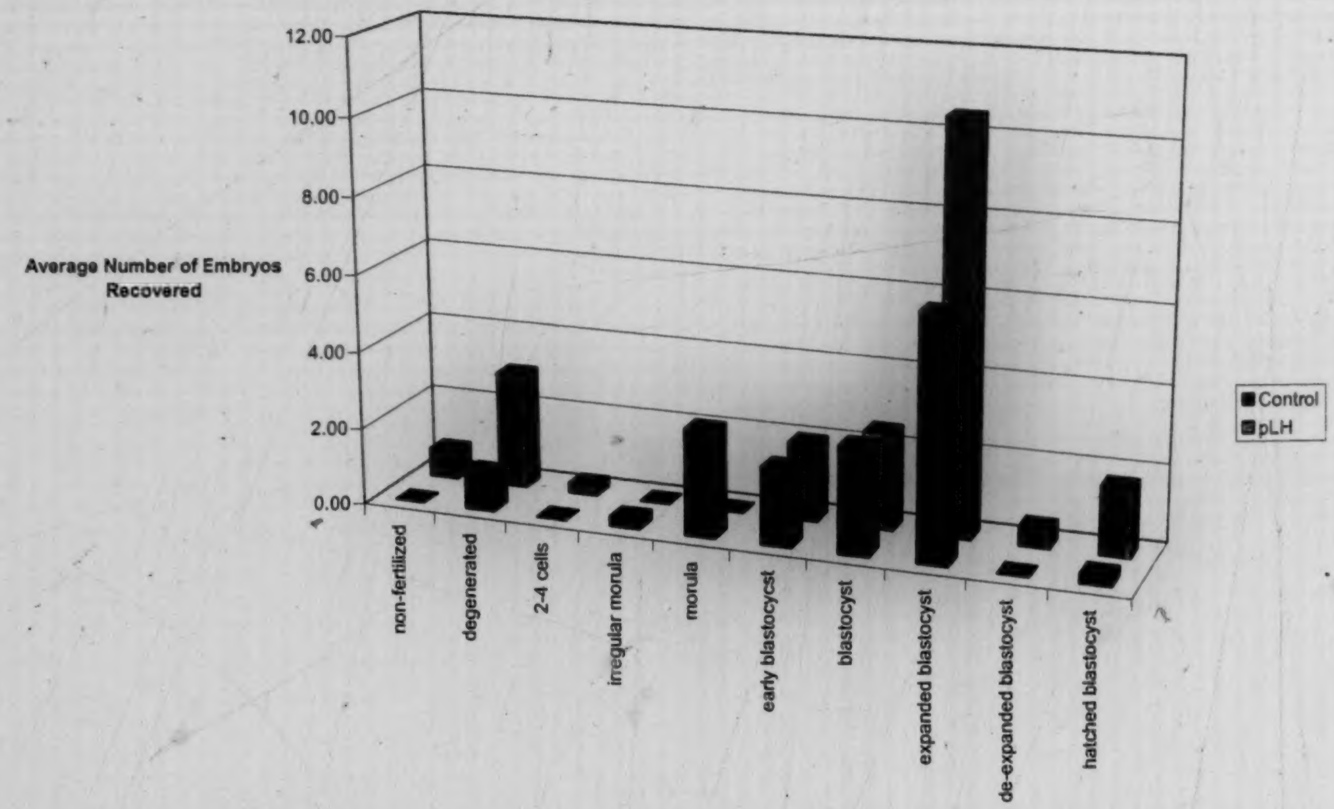

Figure 2.16: Average numbers of embryos recovered at each stage of development for each treatment at day 6.4 (± 0.3) of gestation. It is important to note that one control sow was euthanized one day early (day 4.8).

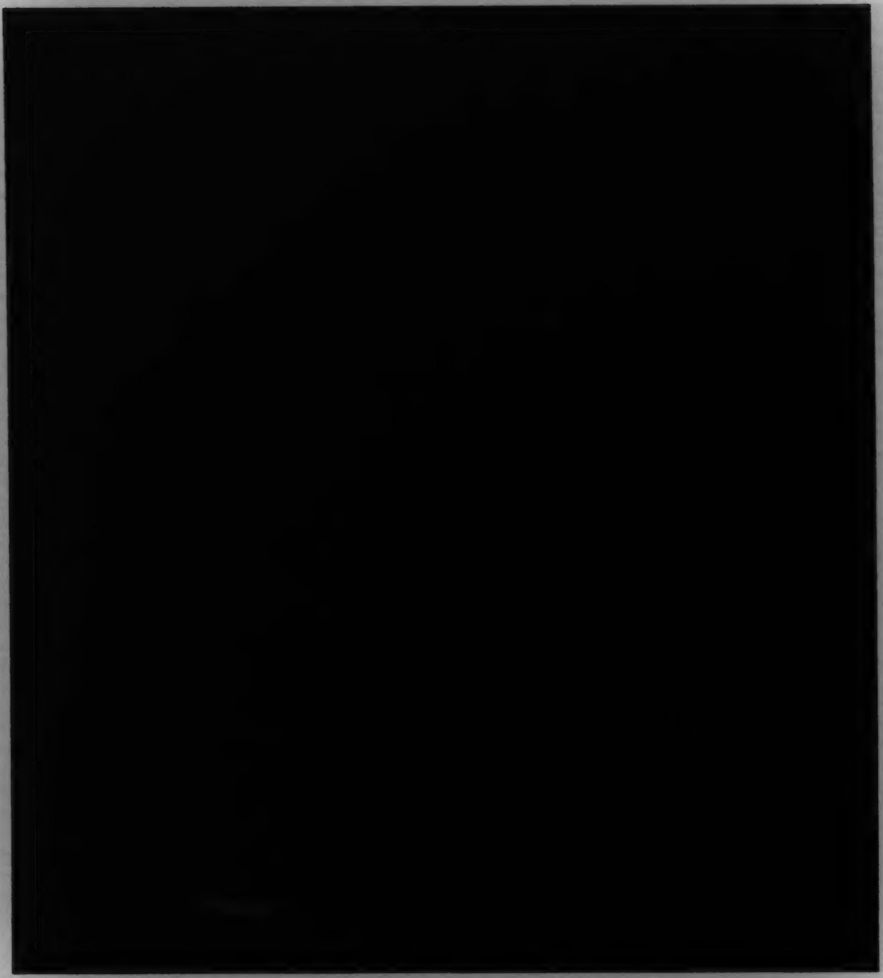

Figure 2.17: An example of a differentially stained expanded blastocyst. Cells appearing pink are stained with propidium iodide, cells appearing blue are stained with bisbenzimide, and cells appearing purple are stained with both.

CHAPTER 3

THE USE OF PORCINE LUTEINIZING HORMONE (pLH) OR HUMAN CHORIONIC GONADOTROPIN (hCG) IN CYCLIC GILTS TO SYNCHRONIZE OVULATION AND THE EFFECT OF THESE TREATMENTS ON EARLY EMBRYO QUALITY

3.1 Introduction

The use of a commercially available porcine luteinizing hormone (pLH) product (Lutropin-V, Bioniche Animal Health Canada Inc., Belleville, Ontario, Canada) in hormonal stimulation protocols has been shown to reliably synchronize ovulation in weaned sows (Candini *et al.* 2001; Viana *et al.* 2002; Cassar *et al.* 2005). In an initial pilot study (Chapter 2), gilts demonstrated a predictable endocrine response to pLH treatment 80 hours after equine chorionic gonadotropin (eCG) treatment, which had initiated follicular development. This study confirmed that pLH treatment usually occurred prior to the endogenous LH surge and induced a significant increase in the peak level (the maximum LH concentration) and amplitude (the increase from baseline LH concentration) of the enhanced LH surge. Furthermore, pLH-treated animals ovulated in a synchronous manner approximately 38 hours after pLH treatment and 33 hours after peak plasma LH concentration was detected.

Hormonal stimulation protocols in swine have typically applied a combination of eCG and human chorionic gonadotropin (hCG) to synchronize estrus and ovulation (Estill 1999). A review of the current literature revealed that there are no studies comparing the synchronizing ability of pLH to hCG. Clearly, it would be advantageous to establish if pLH offers any advantages over this benchmark product, and if pLH can improve the efficacy of fixed-time artificial insemination (AI) protocols. Furthermore, as concerns have been raised regarding the effect of hormonal treatment on embryo quality, examining the quality of the embryos recovered would reveal which hormonal treatment, if any, produces better quality embryos. An effect of hCG has been noted on oocyte maturation (Wiesak *et al.* 1990) which may have implications for embryo quality. Therefore, there are implications not only for embryo survival and litter sizes, but for other reproductive technologies that rely on fixed-time AI protocols, such as embryo transfer.

Heterogeneity of early embryonic development has previously been stated to be an important factor in embryonic loss prior to day 30 of gestation (Geisert and Schmitt 2002). Such embryonic heterogeneity among litters, regardless of any experimental treatment, was recently observed in a study examining the effect of feed restriction during lactation on subsequent oocyte and embryo quality (Vinsky *et al.* 2007). One explanation for variation in developmental stages within a litter is a prolonged duration of the ovulation period (Pope *et al.* 1986), although this concept has been refuted in a study by Soede *et al.* (1992) who found no relationship between the duration of ovulation and embryonic heterogeneity. If duration of ovulation does indeed have an effect on embryo heterogeneity, synchronizing ovulation with exogenous hormones may reduce this variation within litters and in turn potentially reduce embryonic loss.

Recently, pLH has also been used in synchronization protocols involving cattle. One observation from these studies is that pLH treatment increases the level of progesterone released by the corpus luteum (Ambrose *et al.* 2005). It has therefore been suggested that pLH improves the quality of the corpus luteum (CL) produced following ovulation stimulated by the pLH treatment (Ambrose *et al.* 2005). This is significant for cattle, as greater progesterone release has been associated with improved embryonic development and maintenance of pregnancy (Ambrose *et al.* 2005; McNeill *et al.* 2006). A rapid rise in progesterone following the onset of the LH surge has also been associated with the more prolific Meishan sows when compared to Large White sows (Hunter *et al.* 1996). As well, Richards *et al.* (1994) showed that LH can increase progesterone secretion from small luteal cells. Nutritional depression of progesterone concentrations in gilts and weaned sows has also been associated with reduced embryo survival (see review of Foxcroft 1997). Therefore, progesterone concentrations can be influenced by LH and could influence embryo survival.

The aims of this study were to confirm trends seen in the previous pilot study with regards to ovulation timing relative to treatment, and establish whether ovulation in cyclic gilts is synchronized following pLH treatment. Also, a comparison was made to hCG, which is typically what is used in ovulation induction protocols, to determine if pLH provides any benefits over this industry standard. Heterogeneity of embryos recovered was also examined to determine if the different ovulation induction protocols reduce variation in embryonic development within individual litters. Finally, plasma progesterone concentrations at day 6 of gestation were measured to determine if pLH treatment in swine has a similar effect on the CL as it does in cattle.

3.2 Materials and Methods

3.2.1 Animals

This study was conducted at the University of Alberta Swine Research and Technology Centre (SRTC) in accordance with Canadian Council on Animal Care guidelines and was approved by the University of Alberta Faculty Animal Policy Welfare Committee (Protocol DYCK-2006-56). Fifteen sets of littermate gilts (three littermates per set; Hypor F1, Landrace x Large White) that had completed their first estrous cycle were used. Gilt sets were selected based on their weight (difference of 15 kg or less) and the synchrony of their first, naturally occurring, estrus. Animals were housed in individual crates and fed at 1.5 x maintenance, with maintenance adjusted for growth being calculated as 112 x Kcal ME kg^{-1} $BW^{0.75}$ (NRC 1998), where ME is metabolic energy and BW is body weight in kg. Animals were exposed to 12 hours light and 12 hours dark each day. First and second estrus was determined by estrus detection once per day using five minute periods of fence-line contact with two different mature boars.

3.2.2 Altrenogest Treatment

The treatment schedule is outlined in Figure 3.1. Starting on day 3 to 16 of their second or third estrous cycle, animals were given oral altrenogest treatment (Regu-mate^{TM}, 15 mg per day, Intervet Canada Inc., Whitby, Ontario, Canada) for 14 to 18 days to synchronize their subsequent estrus and to allow the animals to fit into the hormonal stimulation protocol.

3.2.3 Hormonal Stimulation Protocol

The day following the end of altrenogest treatment, all gilts were treated with 600 IU eCG (Folligon, Intervet Canada Inc., Whitby, Ontario, Canada) intramuscularly (im). Animals within a litter were then randomly assigned and received either 5 mg pLH im (Lutropin-V, Bioniche Animal Health Canada Inc., Belleville, Ontario, Canada), 750 IU hCG im (Chorulon, Intervet Canada Inc., Whitby, Ontario, Canada), or saline, 80 hours after eCG treatment. The same lot of each hormone was used throughout the experiment to avoid variation.

3.2.4 Determination of Ovulation Timing and Breeding

To determine the onset of estrus and ovulation following hormonal stimulation, estrus detection was performed every eight hours (approximately 8:00 AM, 4:00 PM, 12:00 AM), beginning eight hours prior to pLH/hCG/saline treatment. Fence-line boar exposure was again performed with two boars for 5 minutes minimum per boar. At the same 8-hour frequency as estrus detection, real-time transcutaneous ultrasonography (Pie medial Scanner 200, model 41480, Maastricht, the Netherlands, using a 5.0/7.5 MHz probe, model 401502, Maastricht, the Netherlands) was performed to observe follicular development and the occurrence of ovulation, which was determined by the disappearance of large preovulatory follicles present at the previous ultrasound session. Estimated time of ovulation was determined using a method similar to that described by Almeida *et al.* (2001) adjusted for frequency of ultrasonography. The relationship between duration of estrus and timing of ovulation was determined by dividing the time between onset of estrus and ovulation by the total duration of estrus (% ovulation). The pLH and hCG treated animals were inseminated at fixed times, 32 hours and if ovulation was not yet confirmed, again at 40 hours, after the pLH/hCG treatment. Control animals were inseminated with the same source and dose of semen 16 hours following the initial detection of standing estrus, and then every 24 hours until ovulation was confirmed. Semen was collected and pooled from Duroc boars housed at the same University of Alberta SRTC facility. The insemination doses of 50 ml, were extended in Beltsville thawing solution (Minitube of America, Verona, Wisconsin, USA), and contained 3×10^9

motile and morphologically normal spermatozoa. All inseminations were performed with semen that was less than three days old.

3.2.5 Embryo Collection

At day 5.9 (± 0.03) of gestation (day of ovulation considered to be day 1), animals were euthanized on site. Reproductive tracts were recovered and ovulation rate was determined by counting the number of corpora lutea on each ovary. To ensure any hidden CLs were not missed, several incisions were made in the ovarian tissue.

A retrograde flush using 10-15 ml pre-warmed (~35°C) phosphate buffered saline (PBS) containing 5 mg/ml of bovine serum albumin (BSA) was performed on the oviduct. Following that, the broad-ligament was cut away from the uterus starting at the cervix and moving towards the oviduct. An incision was made at the bottom of the uterine body near its intersection with the cervix, and a modified syringe cylinder with the top remolded was inserted into the incision and secured in place. A 25 ml volume of the PBS-BSA solution was injected into each uterine horn via the oviduct at the utero-tubal junction. The PBS-BSA solution was then pushed through the uterus until it drained out through the modified syringe into a 50 ml plastic conical collection tube (Falcon tube, Becton Dickinson Labware, Franklin Lakes, New Jersey, USA). This procedure was repeated once per uterine horn. The flush solution was stored in pre-warmed, insulated containers to maintain embryo viability.

3.2.6 Embryo Classification and Staining

Embryos were identified under a dissecting microscope at 25 to 50x magnification (Leica MZ 12_5, Leica Microsystems Ltd., Switzerland), harvested, and classified by developmental stage. Embryo classification was based on criteria similar to those described by Machàty *et al.* (1998). Morula stage embryos were those with many blastomeres but no blastocoel. Embryos with a small blastocoel and thick zona pellucida were classified as early blastocysts. Embryos with many blastomeres, a large blastocoel, but a thick zona pellucida were classified as non-expanded blastocysts. Embryos with a very large blastocoel that made the embryo transparent in appearance and a very thin zona pellucida were classified as expanded blastocysts. Embryos that had a visible blastocoel and had clearly stretched the zona pellucida, but had since compacted were classified as de-expanded blastocysts. Embryos that were no longer encased in a zona pellucida, regardless of the degree of expansion, were classified as hatched blastocysts.

All blastocyst stage embryos were viewed under normal bright field microscopy using an inverted microscope (Olympus IX81-UCB-2, Tokyo, Japan) at 200x magnification and three-dimensional images were obtained using Metamorph software (Version 7.1.0.0, Molecular Devices) to determine the degree of expansion.

If expanded blastocysts were present, a subset of these blastocysts from each animal were stained using a modified differential staining procedure utilizing the two fluorochromes propidium iodide and bisbenzimide to visualize trophectoderm (TE) and inner cell mass (ICM) cells (Thouas *et al.* 2001). Briefly, embryos were initially placed in 500 µl of 0.1 mg/ml propidium iodide (P4864, Sigma Aldrich Inc., St. Louis, Missouri) in PBS and 1% Triton-X (T9284, Sigma Aldrich Inc., St. Louis, Missouri) solution, for 15 to 25 seconds and then washed three times in 100% ethanol. Embryos were then transferred to 500 µl of stain 2 containing 0.025 mg/ml bisbenzimide H 33258 trihydrochloride (B2883, Sigma Aldrich Inc., St. Louis, Missouri) in 100% ethanol and incubated overnight at 5°C. Embryos were then washed with 100% ethanol, left in the well to allow for three dimensional imaging, and examined under ultraviolet light at 200x magnification using an inverted microscope (Olympus IX81-UCB-2, Tokyo, Japan) fitted with appropriate filters to evaluate the staining. Metamorph software (Version 7.1.0.0, Molecular Devices) was used to obtain three-dimensional images of each fluorochrome to perform cell counting procedures.

3.2.7 Embryo Scoring

To evaluate embryo homogeneity within a litter, embryos were subjectively scored using the method described by Vinsky *et al.* (2007), similar to the method used by Machàty *et al.* (1998). Briefly, non-fertilized and degenerated embryos were given a score of 2, early stage embryos scored 4, morula stage embryos scored 6, non-expanded blastocysts scored 8, and expanded, de-expanded, and hatched blastocysts scored 10. The sum of the scores for individual embryos within a litter was calculated for each gilt, generating an overall gilt score. Higher scores indicated that a large number of well-developed embryos were recovered. Lower scores indicated poor fertilization, less developed embryos and fewer embryos recovered.

3.2.8 Blood Sample Collection and Progesterone Assay

At euthanasia, 2 to 5 ml blood samples were collected by jugular venipuncture from a subset of animals into heparinized tubes (1000 IU heparin/ml solution, one to two drops per tube). Samples were centrifuged for 15 minutes at 2500g, and the plasma was transferred into separate tubes and stored at approximately –30°C until analysis. Neat plasma was initially assayed in triplicate using the Coat-A-Count® Progesterone radioimmunoassay (Diagnostic Products Corp., Los Angeles, California, USA, Catalogue # TKPG1); however, concentrations of progesterone were too high to be accurately determined in that assay. Therefore, samples were diluted and the assay was rerun. Blood samples from a total of 30 animals were used (10 littermate sets). Plasma samples were diluted 20-fold in assay buffer, and a 100 μl volume of diluted sample was taken to assay in triplicate. Assay sensitivity defined as 89.6% bound was 0.01 ng per tube and all samples were within the sensitivity of the assay. The intra-assay % coefficient of variation was 2.98. A serially diluted plasma pool showed parallelism to the standard curve.

3.2.9 Statistical Analysis

All data were verified for normality and equality of variance using the PROC UNIVARIATE and PROC GLM (Bartlett and Levene tests) procedures in SAS (SAS Institute, Cary, NC, USA, 1990). Data that met the assumptions of typical statistical tests were analyzed using the PROC MIXED procedure. Any others were transformed to a normal distribution and then tested using the PROC MIXED procedure. All proportional data were arcsin transformed to normalize the data and then tested using the PROC MIXED procedure. The design was analyzed as a randomized complete block design with no replication within the blocks. The fixed effect in the model was considered to be the treatment (pLH, hCG, or saline) and the random effect in the model was the pairs of littermates. Length of altrenogest treatment was tested as a covariate and included when significant. The experimental unit was the gilt and, therefore, individual embryo parameters (diameter and total cell count) were averaged within each litter, and the averages were used to evaluate the effect of treatment. All results given are the least square means and standard errors calculated by SAS following PROC MIXED analysis.

3.3 Results

3.3.1 Estrus and Ovulation Timing

There was no difference in age at first estrus (average of 180 ± 9 days), or at the onset of treatment (average of 211 ± 10 days), among treatments. Throughout the trial, all animals remained in a similar metabolic state. The duration of estrus following hormonal stimulation did not differ among the groups, nor did the timing of ovulation in relation to onset of estrus (Table 3.1). However, a littermate effect was observed on duration of standing estrus ($p = 0.01$), which then tended to influence the interval between onset of estrus and ovulation ($p = 0.06$). It should also be noted that one pLH-treated animal did not display standing estrus and one hCG-treated animal and two p-LH treated animals were detected in estrus for only 16 hours.

Ovulation occurred earlier following treatment ($p < 0.001$) in the pLH and hCG groups (43.2 ± 2.5 h and 47.6 ± 2.5 h, respectively) when compared to control animals (59.5 ± 2.5 h), although the duration of standing estrus did not differ (see Table 3.1). The ranges in ovulation timing following treatment were 35.6 to 52.1 h for the pLH group, 36.2 to 67.3 h in the hCG group, and 36.1 to 83.8 h in the control group. Furthermore, overall variance of the treatment-ovulation interval differed among treatments ($p < 0.01$; Figure 3.2), with the pLH treatment group showing the least variation. The average diameter of the largest follicles prior to ovulation was greater ($p = 0.05$) in the control gilts (8.6 ± 0.2 mm) than in the pLH- and hCG-treated animals (8.1 ± 0.2 and 8.1 ± 0.2 mm, respectively).

The majority of the hormonally stimulated gilts received two inseminations (28 out of 30), with the second insemination being very close to the timing of ovulation (Table 3.1). No difference in the timing of the last insemination prior to ovulation was found between control (7.9 ± 1.5 h) and treated animals (3.5 ± 1.5 h and 7.7 ± 1.5 h in pLH- and hCG-treated gilts, respectively).

3.3.2 Progesterone Concentrations

No difference in progesterone concentrations among treatments were detected (Table 3.2). As seen in Figure 3.3, there was variation in circulating progesterone concentrations among individuals; however, overall there was little difference among littermate sets. Plasma progesterone concentrations at day 6 of gestation were positively correlated with ovulation rate ($r = 0.75$, $p < 0.001$, Figure 3.4) with the strongest correlation in the hCG-treated animals (Figure 3.5).

3.3.3 Day 5 Embryo Results

After euthanasia at day 5.9 (± 0.03) of gestation, (day 1 being the day of ovulation) no unfertilized oocytes or embryos were recovered from any oviductal flushings. Ovulation rate, occurrence of ovarian cysts, total number of embryos recovered, and recovery rate of embryos did not differ among treatments (Table 3.2). Ovulation rate ranged from 13 to 32 in control animals, 13 to 40 in pLH-treated animals, and 11 to 32 in hCG-treated animals. Total embryos recovered ranged from 8 to 23 in control animals, 9 to 33 in pLH-treated animals, and 7 to 20 in hCG-treated animals. In contrast, the number of viable embryos recovered (those that were fertilized and not degenerating) tended to be higher in the pLH group (13.3 ± 1.5) when compared to hCG-treated (9.7 ± 1.5; $p = 0.07$) but were not different from control animals (11.2 ± 1.5; $p = 0.29$); the number of viable embryos also tended to be influenced by litter ($p = 0.08$). Viable embryos recovered ranged from 0 to 20 in control animals, 1 to 32 in pLH-treated animals, and 0 to 17 in hCG-treated animals.

3.3.4 Embryo Quality and Embryo Scoring

There were no differences among treatments in the developmental stages of embryos collected at day 5.9 (± 0.03) of gestation (Table 3.3). However, an effect of litter was observed on the number of expanded blastocysts recovered ($p = 0.05$). The total numbers of blastocysts evaluated by developmental stage were as follows: 16 early blastocysts, 64 non-expanded blastocysts, 321 expanded blastocysts (194 of which were used for total cell counts), 21 de-expanded blastocysts, and 60 hatched blastocysts (Figures 3.6 to 3.8). Differential staining of the ICM and TE was not obtained when evaluating the embryos (Figure 3.9), so total cell counts were used. Embryo diameter of unhatched blastocysts and total cell number of expanded blastocysts was not affected by treatment (Table 3.4). Diameters of de-expanded and hatched blastocysts were also evaluated. However, due to high variation in these measurements, resulting from varying degrees of expansion/contraction, these measurements were determined to be unreliable. Regardless of treatment, there was a strong correlation between cell number and diameter of expanded blastocysts (Figure 3.10; $r = 0.88$, $p < 0.0001$). When evaluated within treatment, this relationship was strongest for embryos from control animals (Figure 3.11).

Length of altrenogest treatment was evaluated as a covariate in the statistical model, and was found to be significant for the number of expanded blastocysts recovered ($p = 0.02$). Therefore, the results for expanded blastocyst number presented in Table 3.3 are least square means adjusted for this covariate, with a trend towards recovery of more expanded blastocysts from pLH-treated animals still observed. However, when evaluating the length of altrenogest treatment following luteolysis, assuming natural luteolysis at day 15 of the estrous cycle (Bazer *et al.* 1982), the effect of altrenogest treatment length is no longer seen as it is no longer a significant covariate.

There was little overall heterogeneity in embryonic development (Figure 3.12) and no difference in embryonic heterogeneity scores among treatments (Table 3.4). Most embryos recovered were at the blastocyst stage of development, and no relationship could be established between the number of expanded and hatched blastocysts recovered and ovulation rate (Figure 3.14), or embryo recovery rate (Figure 3.15). Seven litters in total (from three control animals, three hCG-treated animals, and one pLH-treated animal) produced no viable and well-developed blastocysts after recovery.

3.4 Discussion

The results of this study illustrate that treatment with pLH can induce ovulation in cyclic gilts in an efficient and reliable manner. Although the average treatment-ovulation interval for pLH animals is somewhat longer than that observed for weaned sows (Cassar *et al.* 2005; Candini *et al.* 2001; Viana *et al* 2002), there is notable variation among these studies. No significant difference in the average interval between hormone treatment and ovulation was seen in the pLH- and hCG- treated animals and the range in treatment-ovulation interval following pLH treatment is consistent with this previous research. However, the reduced variation in the treatment-ovulation interval for pLH-treated animals compared to either hCG-treated or control animals, is of particular interest. From a practical perspective this indicates that the timing of ovulation was more consistent following pLH treatment. This is a major consideration for fixed-time AI protocols which use specific AI timing following hormone treatment; as a result, pLH-treated animals would be more likely to receive a properly timed insemination. In the previous pilot study, the second insemination tended to occur after ovulation in most cases and was considered redundant. In the current study, this was not an issue and, therefore, the two fixed-time inseminations used in this study were appropriate following pLH treatment. Furthermore, Soede *et al.* (1995) reported that insemination up to 8 hours after ovulation did not detrimentally affect fertilization rates; therefore, in future situations where timing of ovulation is not being monitored and an insemination does occur shortly after ovulation, there should be no detrimental effects on subsequent fertility.

The reduced variability in treatment-ovulation interval in pLH-treated gilts highlights the possibility for the use of pLH in fixed-time insemination protocols utilizing a single insemination. Treatment with pLH stimulates ovulation in a predictable manner, allowing for an insemination to be scheduled based on the timing of treatment. Although there is some variation in response to treatment among animals, the variation is small enough to allow for one insemination to be given very close to the time of ovulation, which is ideal for successful fertilization. For animals in which the stage of the estrous cycle is known, pLH could be given late into the follicular phase (~d18) to induce ovulation. When the status of the animal is unknown, pLH treatment should be combined with other synchronization techniques, such as altrenogest, to ensure proper timing of pLH treatment. In a production based setting, an example of a possible protocol would to begin altrenogest treatment for 14 days on the Friday two weeks prior to anticipated eCG treatment, withdraw it on Thursday, give eCG at 4 PM on Friday, followed by pLH injection 4 PM Monday, with inseminations as early as possible on Tuesday. However, due to the manipulation of the interval between eCG and pLH, this protocol should be evaluated prior to being introduced into a production herd to ensure it is reliable. Also, boar exposure during this time should be considered, as the effect of removing the boar during the stimulation period was not tested in this study.

Treatment had no effect on plasma progesterone concentrations measured at day 6 of gestation, and although progesterone concentrations were influenced by ovulation rate, ovulation rate did not differ among treatments. The relationship between the number of CLs and progesterone levels is similar to that observed by Knox *et al.* (2003). Therefore, it does not appear that pLH has any effect on the capacity of the resulting CLs to produce progesterone at this stage of gestation. However, the time of sampling in this study may not have been ideal to allow for detection of differences in progesterone levels. A previous study, looking at endocrine differences between Large White and Meishan breeds, measured progesterone in the immediate period following ovulation and found that the rate of progesterone increase may be an important indicator for subsequent embryonic survival (Hunter *et al.* 1996). Smaller and delayed increases in the progesterone concentration in the 72-hour period following onset of estrus in gilts and weaned sows have been associated with reduced embryonic survival following nutritional restriction (reviewed by Foxcroft 1997). Peripheral progesterone concentrations appear to increase beginning approximately 40 hours after the peak of the LH surge and begin to reach a plateau by 100 hours following the peak (Novak *et al.* 2003). By day 6 of gestation, these differences may no longer be detectable.

Evaluating the diameter of the largest follicle present prior to ovulation in this study showed that hormonal stimulation induces smaller follicles to ovulate when compared to the follicle size in control animals, possibly due to earlier ovulation. This brings into question the potential quality of the oocytes and embryos resulting from this protocol. With this in mind, the effect of hormone treatment on resulting embryo quality was also evaluated, particularly given that hormone treatment protocols are an important component of embryo transfer procedures. Despite these concerns, Faillace and Hunter (1994) found that the more prolific Meishan breed tends to have smaller follicles than the Large White breed, yet the oocytes are more developmentally advanced. Therefore, the difference between treatment groups in follicular size may not necessarily be a factor in oocyte quality. Various approaches have been used to evaluate embryonic quality including: morphological evaluation; rate of development; cell counting; and evaluating rates of apoptosis (Almeida *et al.* 2001; Fouladi-Nashta *et al.* 2005; Ock *et al.* 2007). Differential staining of the ICM and TE cells has been established for bovine and mouse embryos (Thouas *et al.* 2001; Fouladi-Nashta *et al.* 2005) and has also been applied to porcine embryos (Machàty *et al.* 1998; Kim *et al.* 2005). In our hands, effective embryonic staining was obtained; however, consistent differential staining of the ICM and TE was difficult to achieve. It has been determined with mouse embryos (George Thouas, personal communication) that differential staining is not efficient for embryos with fewer than 100 blastomeres. Given that the majority of the expanded blastocysts evaluated in this study had total cell counts of less than 100, this may explain our limited ability to distinguish between ICM and TE cells. On the other hand, Kim *et al.* (2005) was able to distinguish between the two types of cells when they stained *in vitro* produced porcine embryos that ranged from approximately 45 to 57 total cells. The staining procedure was very similar to the one used in the present study, however, the embryos were mounted and there is no description of how the cells were counted. Therefore, the differing results among the studies may be due to differences in counting technique. It is possible, that by mounting the embryos, some cells may have been missed. Another study that was also able to distinguish between the ICM and TE cells in porcine embryos used rabbit anti-pig serum to disrupt the TE, possibly providing a more specific means of permeating the outer trophectoderm cells (Tao *et al.* 1995)

Regardless, total cell counts have been used on numerous occasions to evaluate embryo quality (Hazeleger *et al.* 2000), including studies that also evaluated ICM and TE cell numbers (Machàty *et al.* 1998; Kim *et al.* 2005; Ock *et al.* 2007). In the present study, total cell counts for expanded blastocysts did not differ among treatments, suggesting that embryo quality was not compromised by hormonal stimulation, even though the size of the follicles being ovulated was smaller. This is encouraging for those who employ embryo transfer procedures, as hormonal control of estrus and ovulation are inherent components of these protocols. It is interesting to note that the total cell counts obtained here differ greatly from other studies involving porcine blastocysts. Ock *et al.* (2007) had total cell counts ranging from 3 to 80, with an average of 29.7, Koo *et al.* (2005) had average cell counts ranging from 34.9 to 44.9, and Machàty *et al.* (1998) reported an average total cell count of 25.3. However, these studies involved *in vitro* produced embryos which may account for these differences. Furthermore, although the cell count totals for *in vitro* derived blastocysts were quite low, Machàty *et al.* (1998) demonstrated that some of these embryos formed conceptuses by day 40 of pregnancy. When reviewing literature involving *in vivo* produced blastocysts, similar total cell numbers are reported to those observed in the present study. Tao *et al.* (1995) reported an average of 74.7 total cells (ranging from 39 to 109 cells overall) and Machàty *et al.* (1998) reported an average total cell number of 54.9 for control *in vivo* produced blastocysts.

It was also determined that expanded blastocyst diameter correlates well with total cell number. However, this relationship may not extend as effectively to de-expanded or hatched blastocysts. During the present experiments, it was seen that the degree of expansion could change within a matter of hours and the amount of expansion seen among de-expanded and hatched blastocysts could vary greatly. Different patterns of hatching in mouse blastocysts have been described by Niimura and Fuji (1997) which clearly illustrates the degree of expansion and contraction experienced by blastocysts at the time of hatching. Therefore, depending on diameter measurements as a predictor of cell number may not be reliable at these later stages of preimplantation development. However, given that the International Embryo Transfer Society (IETS) recommends the transfer of zona pellucida intact embryos (Stringfellow and Seidel 1998), the correlation between expanded blastocyst diameter and total cell number is very relevant.

Although quality of blastocysts obtained from these animals was not affected by treatment, there was an overall trend indicating that fewer viable embryos were obtained from hCG-treated animals, especially when compared to pLH-treated animals. This is consistent with previous research indicating that hormone treatment may reduce embryo viability (Ziecik *et al.* 2005; do Lago *et al.* 2005). It was also noted that hCG increased variability in the stage of oocyte maturation following treatment (Wiesak *et al.* 1990), which may influence the viability of these oocytes once they are fertilized. This difference between hormone-treated groups could be related to the fact that pLH is an endogenous hormone in swine, and as a result, may have fewer detrimental effects than a hormone that is not naturally found in these animals. As well, pLH has a relatively short half-life in circulation (Esbenshade *et al.* 1986), so it rapidly influences follicular growth and ovulation but is then cleared quickly. In comparison, hCG remains in circulation longer (Stone *et al.* 1987), which is a possible explanation as to why there is more variation in the treatment-ovulation interval observed with this treatment. Because a decrease in viability does not appear to occur in the pLH-treated group, it may be advantageous to use pLH in synchronization protocols rather than hCG.

A recent study by Vinsky *et al.* (2007), involving feed restriction in lactating sows, identified a subpopulation of animals which produce very heterogeneous litters in regard to embryo development independent of treatment. This finding was not supported by the present study as all treatments were quite homogenous with regard to embryo development (Figure 3.12). Certain animals (three control animals, three hCG-treated animals, and one pLH-treated animal) did not produce expanded blastocysts, but this was due to a lack of successful fertilization rather than high heterogeneity, and this tended to be influenced by the litter (of the 7 animals, 6 were out of 3 sets of littermates). In only two cases, there was no evidence of sperm (no fertilization or no sperm present in the zona pellucida), and all animals had properly timed inseminations and large pre-ovulatory follicles. There was variation in length of altrenogest treatment, as well as duration of estrus, and timing of ovulation with relation to that duration among the gilts. Therefore, it is difficult to explain this failure in successful fertilization. However, although this effect was only observed in a small percentage of animals, these results may highlight a subpopulation of gilts with poor reproductive potential, which therefore should be culled. Given that all animals evaluated had relatively high scores related to embryonic heterogeneity, no 'low' animals such as those seen in Vinsky *et al.*'s study (2007) could be defined here. This indicates that the majority of the animals had litters containing embryos at similar, advanced stages of development (expanded or hatched blastocyst).

Vinsky *et al.* (2007) also found a relationship between the number of advanced blastocysts recovered and the overall embryo recovery rate. It was unclear if this effect was due to the possibility these unrecovered embryos remained in the oviduct and could not be collected by uterine flush, or whether it was a true loss or destruction of the embryos that were not recovered. In the present study, retrograde flushing of the oviduct did not recover any oocytes or embryos. This indicates that embryos not recovered by uterine flushing were not missed because they were in the oviduct. Furthermore, unlike the Vinsky *et al.* (2007) study, significant homogeneity within litters among gilts was seen in the present study, with most animals producing embryos at an advanced stage of development. Notably, only 3 animals had recovery rates under 60%, so overall the embryos recovered in this present study would appear to be representative of what was present in the litter and embryo loss was not a significant factor. Furthermore, sub-populations of animals were defined in terms of the number of expanded and hatched blastocysts recovered by Vinsky *et al.* (2007). Within these populations, a relationship between the number of advanced blastocysts recovered and ovulation rate was observed. In the present study, when examined overall, there was no relationship between ovulation rate and the number of advanced blastocysts recovered. This is to be expected as follicular growth was being exogenously induced and animals were not in any way compromised prior to or during treatment, so ovulation rates consistently exceeded 10. With regard to the population of animals who had ovulation rates from 15 to 25, but had 10 or fewer expanded and hatched blastocysts, this was either due to a low embryo recovery rate or the presence of blastocyst stage embryos that had not yet expanded.

As discussed, although the interval to ovulation in the pLH-treated animals was less variable, this did not translate into differences among treatments in embryo heterogeneity. However, a trend toward more viable embryos and higher numbers of expanded blastocysts in pLH-treated animals was observed and use of pLH in embryo transfer protocols may, therefore, be advantageous. Notably, one pLH-treated animal was observed to have an ovulation rate of 40, from which 32 embryos were collected, and of those, 27 were expanded blastocysts. High ovulation rates (greater than 25) were also observed in some hCG-treated and control animals, with the majority of these animal producing high numbers of well-developed embryos as well (15 to 19). In a situation where fixed-time AI is being used for production purposes, this could have implications for uterine crowding if pregnancies such as these are allowed to carry to term. Placental weight and volume can be decreased with only slight crowding within the uterus and can lead to intra-uterine growth retardation and negatively affect myogenesis in the fetus (Foxcroft *et al.* 2007). Variation in ovulation rate was emphasized in a study by Fujino *et al.* (2006), who found a dosage of 1500 IU eCG and 500 IU hCG in prepubertal gilts resulted in ovulation rates ranging from 6 to 72. Clearly there is large individual variation in how animals respond to the hormone treatments, particularly eCG. Therefore, eCG dosage may need to be limited in protocols where such extreme ovulation rates are not ideal but even with a low dose of eCG (as used in this study), these high rates of ovulation may still occur.

An unexpected effect of altrenogest treatment was observed. In previous studies, reproductive characteristics have not been affected by altrenogest treatment when duration of treatment is 14 days or greater (Estill 1999). Therefore, no effect of altrenogest treatment was expected. However, when this parameter was included as a covariate in the analysis, it was seen to influence the number of expanded blastocysts recovered. There was no difference between 14-day and 15-day treatments or 15- and 18-day treatments, but expanded blastocyst number was significantly lower for an 18-day treatment when compared to a 14-day treatment (p=0.02). Although previous research indicates altrenogest treatment prevents follicular growth (Guthrie and Bolt 1985), it is possible that some development did occur during this period, resulting in 'aged' oocytes being present at the time of ovulation induction. On the other hand, when evaluating the actual number of days that animals were on altrenogest treatment following natural luteolysis as a covariate, there is no longer any effect. Therefore, it appears as though there may be compounding factors involved, including litter effects, and altrenogest treatment may not be influencing the number of expanded blastocysts recovered. As well, only 9 animals received an 18-day altrenogest treatment and only 7 animals received a 15-day treatment, so it may not be reliable to draw definitive conclusions on the effect of altrenogest treatment length based on this study. Nonetheless, because this can have implications for any synchronization protocol utilizing altrenogest to produce large numbers of viable embryos, the length of altrenogest treatment may need to be restricted. Despite this, there appears to be a tendency for greater numbers of expanded blastocyst to be recovered from the pLH-treated animals as seen in Table 3.3.

Overall, this experiment confirmed that pLH is an appropriate product to use in synchronization protocols for cyclic gilts, resulting in consistent and reliable timing of ovulation following treatment. Reduced variability in the treatment-ovulation interval, as well as the tendency to produce a greater number of viable embryos are both benefits of the pLH treatment in contrast to hCG. These results also illustrate the potential of this product for use in fixed-time AI protocols in which only one insemination is given. A lack of any detectable effects on embryo quality is also encouraging for embryo transfer procedures in which large numbers of well-developed, viable embryos are required. The use of pLH in these situations should be explored further, in order to establish reliable techniques that can be used in production settings.

3.5 References

Almeida, F.R.C.L., Mao, J., Novak, S., Cosgrove, J.R., Foxcroft, G.R. 2001. Effects of different patterns of feed restriction and insulin treatment during the luteal phase on reproductive, metabolic, and endocrine parameters in cyclic gilts. *J. Anim. Sci.* 79:200–212.

Ambrose, J.D., Kastelic, J.P., Rajamahendran, R., Aali, M., Dinn, N. 2005. Progesterone (CIDR)-based timed AI protocols using GnRH, porcine LH or estradiol cypionate for dairy heifers: Ovarian and endocrine responses and pregnancy rates. *Theriogenology.* 64:1457–1474.

Bazer, F.W., Geisert, R.D., Thatcher, W.W., Roberts, R.M. 1982. The establishment and maintenance of pregnancy. In Control of Pig Reproduction. Cole, D.J.A, Foxcroft, G.R. Eds. pp. 227-252.

Candini, P.H., Moretti, A.S., Silveria P.R.S., Zanella, E., Viana, C.H.C., Santos, I.C.C. 2001. Utilization of a protocol of synchronization of ovulation, induced by porcine LH, to perform insemination programs in fixed time, with one or two inseminations per sow. Sixth International Conference on Pig Reproduction, Columbia MO. pp. 99.

Cassar, G., Kirkwood, R.N., Bennett-Steward, K., Friendship, R.M. 2005. Towards timed single insemination in sows. Proceedings of the 36th Annual Meeting of the American Association of Swine Veterinarians, Toronto, ON. pp. 353-356.

do Lago, V., Viana, W.L., Gama, R.D., de Campos Rosseto, A., Pinese, M.E., de Sant'Anna Moretti, A. 2005. Second oestrus synchronization and precocious embryo viability after puberty induction in gilts by the use of gonadotrophin treatment. *Reprod. Dom. Anim.* 40:141–144.

Estill, C.T. 1999. Current concepts in estrus synchronization in swine. Proceedings of the American Society of Animal Science. Southern Section ASAS Meeting, Memphis, TN. E38.

Faillace, L.S., Hunter, M.G. 1994. Follicle development and oocyte maturation during the immediate preovulatory period in Meishan and white hybrid gilts. *J. Reprod. Fert.* 101:571-76.

Fouladi-Nashta, A.A., Alberio, R., Kafi, M., Nicholas, B., Campbell, K.H.S., Webb, R. 2005. Differential staining combined with TUNEL labeling to detect apoptosis in preimplantation bovine embryos. Reproductive BioMedicine Online. 10:497-502.

Foxcroft, G.R. 1997. Mechanisms mediating nutritional effects on embryonic survival in pigs. *J. Reprod. Fert. Suppl.* 52:47-61.

Foxcroft, G.R., Vinsky, M.D., Paradis, F., Tse, W-Y., Town, S.C., Putman, C.T., Dyck, M.K., Dixon, W.T. 2007. Macro-environment effects on oocytes and embryos in swine. *Theriogenology.* Epub.

Fujino, Y., Nakamura, Y., Kobayashi, H., Kikuchi, K. 2006. Relationship between time elapsed after human chorionic gonadotropin administration and developmental stage in porcine embryos collected from prepubertal gilts. *J. Reprod. Dev.* 52:267-275.

Geisert, R.D., Schmitt, R.A.M. 2002. Early embryonic survival in the pig: Can it be improved? *J. Anim. Sci.* (E. Supplement 1) 80:E54-E65.

Guthrie, H.D. 2005. The follicular phase in pigs: Follicle populations, circulating hormones, follicle factors, and oocytes. *J. Anim. Sci.* 83(Suppl.):E79-89.

Guthrie, H.D., Bolt, D.J. 1985. Pituitary and ovarian hormone secretion and ovulation in gilts injected with gonadotropins during and after oral administration of progesterone agonist (Altrenogest). *Biol. Repro.* 33:679-689.

Hazeleger, W., Bouwman, E.G., Noordhuizen, J.P.T.M., Kemp, B. 2000. Effect of superovulation induction on embryonic development on day 5 and subsequent development and survival after nonsurgical embryo transfer in pigs. *Theriogenology.* 53:1063-1070.

Hunter, M.G., Picton, H.M., Biggs, C., Mann, G.E., McNeilly, A.S., Foxcroft, G.R. 1996. Periovulatory endocrinology in high ovulating Meishan sows. *J. Endocrinol.* 150:141-47.

Kim, H-s., Lee, G-s., Hyun, S-h., Nam, D-h., Lee, S-h., Jeong, Y-W., Kim, S., Kim, J-h., Kang, S-k., Lee, B-c., Hwang, W-s. 2005. Embryotropic effect of glycosaminoglycans and receptors in development of porcine pre-implantation embryos. *Theriogenology.* 63:1167-1180.

Koo, D-B., Kim, Y-J., Yu, I., Kim, H-N., Lee, K-K., Han, Y-M. 2005. Effects of in vitro conditions on preimplantation development and quality of pig embryos. *Anim. Reprod. Sci.* 90:101-10.

Knox, R.V., Vatzias, G., Naber, C.H., Zimmerman, D.R. 2003. Plasma gonadotropins and ovarian hormones during the estrous cycle in high compared to low ovulation rate gilts. *J. Anim. Sci.* 81:249-60.

Liu, Z.H., Yue, K.Z., Ma, S.F., Sun, X.S., Tan, J.H. 2003. Effects of pregnant mare serum gonadotropin (eCG) on follicle development and granulosa-cell apoptosis in the pig. *Theriogenology.* 59:775-785.

Machàty, Z., Day, B.N., Prather, R.S. 1998. Development of early porcine embryos in vitro and in vivo. *Biol. Reprod.* 59:451-55.

Martinat-Botte, F., Bariteau, F., Forgerit, Y., Macar, C., Moreau, A., Terqui, M., Signoret, J.P. 1990. Control of oestrus in gilts II. Synchronization of oestrus with a progestagen, altrenogest (regumate): Effect on fertility and litter size. *Anim. Reprod. Sci.* 22:227-33.

Martinat-Botte, F., Bariteau, F., Forgerit, Y., Macar, C., Poirier, P., Terqui, M. 1995. Synchronization of oestrus in gilts with altrenogest: effects on ovulation rate and foetal survival. *Anim. Reprod. Sci.* 39:267-74.

McNeill, R.E., Diskin, M.G., Sreenan, J.M., Morris, D.G. 2006. Associations between milk progesterone concentration on different days and with embryo survival during the early luteal phase in dairy cows. *Theriogenology.* 65:1435-1441.

Niimura, S., Fuji, M. 1997. A morphological study of blastocyst hatching in the mouse and rat. *J. Reprod. Dev.* 43:295-302.

Novak, S., Almeida, F.R.C.L., Cosgrove, J.R., Dixon, W.T., Foxcroft, G.R. 2003. Effect of pre- and postmating nutritional manipulation on plasma progesterone, blastocyst development, and the oviductal environment during early pregnancy in gilts. *J. Anim. Sci.* 81:772-83.

NRC. 1998. *Nutritional Requirements of Swine.* Washington, DC, National Academy Press.

Ock, S-A., Lee, S-L., Kim, J-G., Kumar, B-M., Balasubramanian, S., Choe, S-Y., Rho, G-J. 2007. Development and quality of porcine embryos in different culture system and embryo-producing methods. *Zygote.* 15:1-8.

Pope, W.F., Lawyer, M.S., Nara, B.S., First, N.L. 1986. Effect of asynchronous superinduction on embryo survival and range of blastocyst development in swine. *Biol. Reprod.* 35:133-137.

Richards, R.G., Gadsby. J.E., Almond, G.W. 1994. Differential effects of LH and PGE_2 on progesterone secretion by small and large luteal cells. *J. Reprod. Fert.* 102:27-34.

Soede, N. M., Noordhuizen, J.P.T.M., Kemp, B. 1992. The duration of ovulation in pigs, studied by transrectal ultrasonography, is not related to early embryonic diversity. *Theriogenology.* 38:653-666.

Soede, N.M., Wetzels, C.C.H., Zondag, W., Hazeleger, W., Kemp, B. 1995. Effects of a second insemination after ovulation on fertilization rate and accessory sperm count in sows. *J. Reprod. Fert.* 105:135-40.

Stringfellow, D.A., Seidel, S.M. 1998. Manual of the International Embryo Transfer Society. 3rd Ed. 309 West Clark Street Champaign, Illinois

Stone, B.A., Heap, P.A., Seamark, R.F. 1987. Changes in peripheral progestagen levels in early pregnant gilts following injection of human chorionic gonadotrophin. *J. Endocrinol.* 115:161-167.

Tao, T., Reichelt, B., Niemann, H. 1995. Ratio of inner cell mass and trophoblastic cells in demi- and intact pig embryos. *J. Reprod. Fert.* 104:251-58.

Thouas, G., Korfiatis, N.A., French, A.J., Jones, G.M., Trounson, A.O. 2001. Simplified technique for differential staining of inner cell mass and trophectoderm cells of mouse and bovine blastocysts. Reproductive BioMedicine Online. 3:25-29.

Viana, C.H.C., Candini, P.H., Gama, R.D., Carbone, A., Santos, I.C.C., Vianna, W.L., Barnabe, R.C. 2002. Evaluation of a synchronization of ovulation protocol in cyclic sows. Proceedings of the International Pig Veterinary Congress, Ames, Iowa #678.

Vinsky, M.D., Paradis, F., Dixon, W.T., Dyck, M.K., Foxcroft, G.R. 2007. Ontogeny of metabolic effects on embryonic development in lactating and weaned primiparous sows. *Reprod. Fertil. Dev.* 19:603-611.

Wiesak, T., Hunter, M.G., Foxcroft, G.R. 1990. Differences in follicular morphology, steroidogenesis and oocyte maturation in naturally cyclic and PMSG/hCG-treated prepubertal gilts. *J. Reprod. Fert.* 89:633-41.

Ziecik, A.J., Biallowicz, M., Kaczmarek, M., Demianowicz, W., Rioperez, J., Wasielak, M., Bogacki, M. 2005. Influence of estrus synchronization of prepubertal gilts on embryo quality. *J. Reprod. Dev.* 51:379-384.

Table 3.1: Estrus characteristics and timing of standing estrus and ovulation following pLH, hCG, or saline treatment (Least square means ± SEM)

	CONTROL **(n = 15)**	**pLH** **(n = 15)**	**hCG** **(n = 15)**
[1]Age at first estrus (d)	180.9 ± 2.4	177.7 ± 2.4	180.5 ± 2.4
Age at Treatment (d)	211.9 ± 2.5	210.1 ± 2.5	211.9 ± 2.5
Weight at onset of estrus after treatment (kg)	137.7 ± 2.3	137.2 ± 2.3	141.5 ± 2.3
Interval – Onset of estrus to altrenogest treatment (d)	5.2 ± 0.2	4.8 ± 0.2	5.0 ± 0.2
Duration of estrus (h) after treatment	48.9 ± 4.1	43.4 ± 4.2	41.5 ± 4.1
Interval - Estrus to ovulation (h) after treatment	38.8 ± 3.5	32.7 ± 3.6	32.8 ± 3.5
% Onset of estrus to ovulation	80.6 ± 5.7	73.9 ± 5.9	81.8 ± 5.7
Interval - First insemination to ovulation (h)	17.6 ± 2.3	11.0 ± 2.3	15.2 ± 2.3
Interval - Second insemination to ovulation (h)	7.1 ± 2.4	3.5 ± 1.6	8.0 ± 1.6
Interval - Last insemination to ovulation (h)	7.9 ± 1.5	3.5 ± 1.5	7.7 ± 1.5
Interval - Treatment to onset of estrus (h)	20.7 ± 4.4	11.0 ± 4.6	14.8 ± 4.4
Interval - eCG to ovulation (h)*	**134.8 ± 4.3**^{a}	**121.7 ± 4.3**^{b}	**122.9 ± 4.3**^{b}
Interval – saline/pLH/hCG to ovulation (h)**	**59.5 ± 2.5**^{c}	**43.2 ± 2.5**^{d}	**47.6 ± 2.5**^{d}
Largest measured follicle (mm)*	**8.6 ± 0.2**^{a}	**8.1 ± 0.2**^{b}	**8.1 ± 0.2**^{b}

*$p<0.05$; ** $p<0.001$; ^{a,b} values with different superscripts are significantly different ($p<0.05$); ^{c,d} values with different superscripts are significantly different ($p=0.001$)
Note: One pLH-treated animal did not display estrus; therefore, 14 pLH-treated animals were used for the analysis of estrus characteristics.

[1]- Stimulation with mature boars commenced at approximately 167 days of age

Table 3.2: Gilt reproductive performance at day 5.9 (± 0.03) of gestation (Least square means ± SEM)

	CONTROL (n = 15)	**pLH** (n = 15)	**hCG** (n = 15)
Weight at euthanasia (kg)	143.1 ± 2.1	139.2 ± 2.1	145.1 ± 2.1
Day of gestation	6.0 ± 0.1	5.9 ± 0.1	5.9 ± 0.1
Ovulation rate	19.3 ± 1.5	20.3 ± 1.5	17.5 ± 1.5
Ovarian cysts	0.7 ± 0.4	0.5 ± 0.4	0.8 ± 0.4
Total embryos recovered	15.3 ± 1.2	16.1 ± 1.2	13.7 ± 1.2
Viable embryos recovered	11.2 ± 1.5	13.3 ± 1.5	9.7 ± 1.5
% Embryo recovery	80.7 ± 3.9	78.8 ± 3.9	78.9 ± 3.9
Progesterone (ng/ml; n = 30)*	20.4 ± 1.6	17.9 ± 1.6	20.0 ± 1.6

*Significant covariate (ovulation rate) $p < 0.001$

Table 3.3: Average number of embryos, by stage of development, recovered at day 5.9 (± 0.03) of gestation (Least square means ± SEM)

	CONTROL (n = 15)	**pLH** (n = 15)	**hCG** (n = 15)
Non-fertilized	3.7 ± 1.5	2.1 ± 1.5	2.9 ± 1.5
Degenerated	0.3 ± 0.3	0.3 ± 0.3	0.4 ± 0.3
Early embryos (2-4 cell to morula)	0.9 ± 0.4	0.3 ± 0.4	0.3 ± 0.4
Early blastocysts	0.5 ± 0.2	0.3 ± 0.2	0.5 ± 0.2
Non-expanded blastocysts	1.3 ± 0.6	1.9 ± 0.6	1.1 ± 0.6
Expanded blastocysts*	6.9 ± 1.4	9.4 ± 1.4	6.6 ± 1.4
De-expanded blastocysts	0.1 ± 0.2	0.9 ± 0.2	0.4 ± 0.2
Hatched blastocysts	1.9 ± 0.9	0.7 ± 0.9	1.6 ± 0.9
Expanded and hatched blastocysts*	8.8 ± 1.5	10.9 ± 1.5	8.6 ± 1.5
Number of litters with expanded, de-expanded, and/or hatched blastocysts	12/15	14/15	12/15

*significant covariate (altrenogest treatment) $p = 0.02$

Table 3.4: Embryo characteristics as measured using Metamorph (Least square means ± SEM); number in brackets indicates the number of animals overall used in the analysis

	CONTROL	**pLH**	**hCG**
Early blastocyst diameter (µm; n = 10)	116.8 ± 8.0	114.7 ± 8.0	119.5 ± 6.9
Non-expanded blastocyst diameter (µm; n = 17)	159.3 ± 6.0	151.2 ± 6.0	155.8 ± 6.6
Expanded blastocyst diameter (µm; n = 36)	190.0 ± 7.7	189.2 ± 6.8	193.3 ± 7.7
Total expanded blastocyst cell number (n = 35)	64.3 ± 6.7	62.9 ± 5.7	67.9 ± 6.4
[1]Litter score	114.4 ± 14.0	136.8 ± 14.0	106.7 ± 14.0

[1]-Litter score is the total of individual embryo scores within a litter; scoring was based on method from Vinsky *et al.* 2007

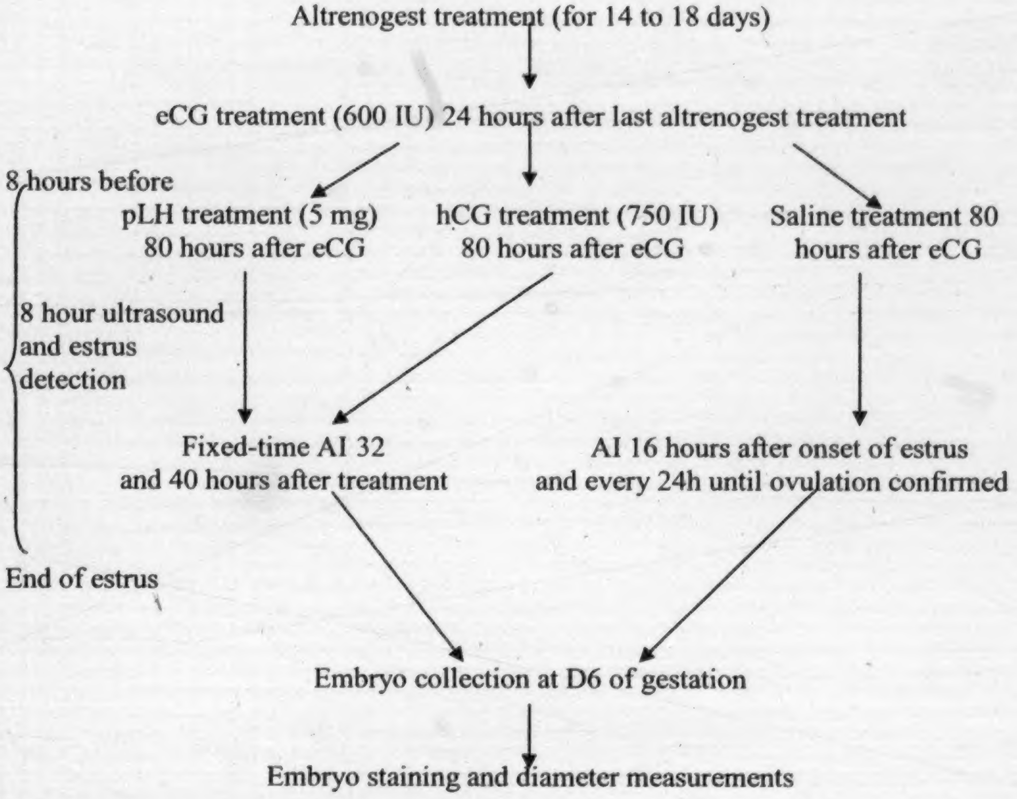
Altrenogest treatment (for 14 to 18 days)
eCG treatment (600 IU) 24 hours after last altrenogest treatment
8 hours before
pLH treatment (5 mg) 80 hours after eCG
hCG treatment (750 IU) 80 hours after eCG
Saline treatment 80 hours after eCG
8 hour ultrasound and estrus detection
Fixed-time AI 32 and 40 hours after treatment
AI 16 hours after onset of estrus and every 24h until ovulation confirmed
End of estrus
Embryo collection at D6 of gestation
Embryo staining and diameter measurements

Figure 3.1: Outline of the treatment schedule.

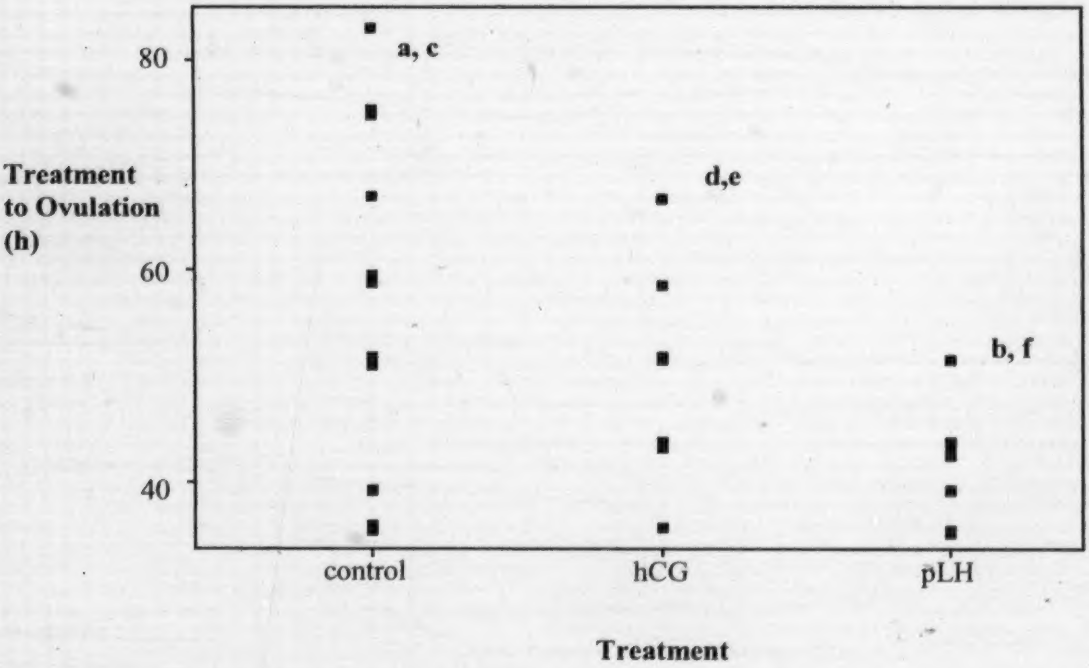

Figure 3.2: Variation in timing of ovulation following saline, hCG, or pLH treatment (Figure generated in SAS and edited for format).
^{a,b} values with different superscripts are significantly different (p = 0.001); ^{c,d} values with different superscripts are significantly different (p = 0.03); ^{e,f} values with different superscripts are different (p = 0.06)

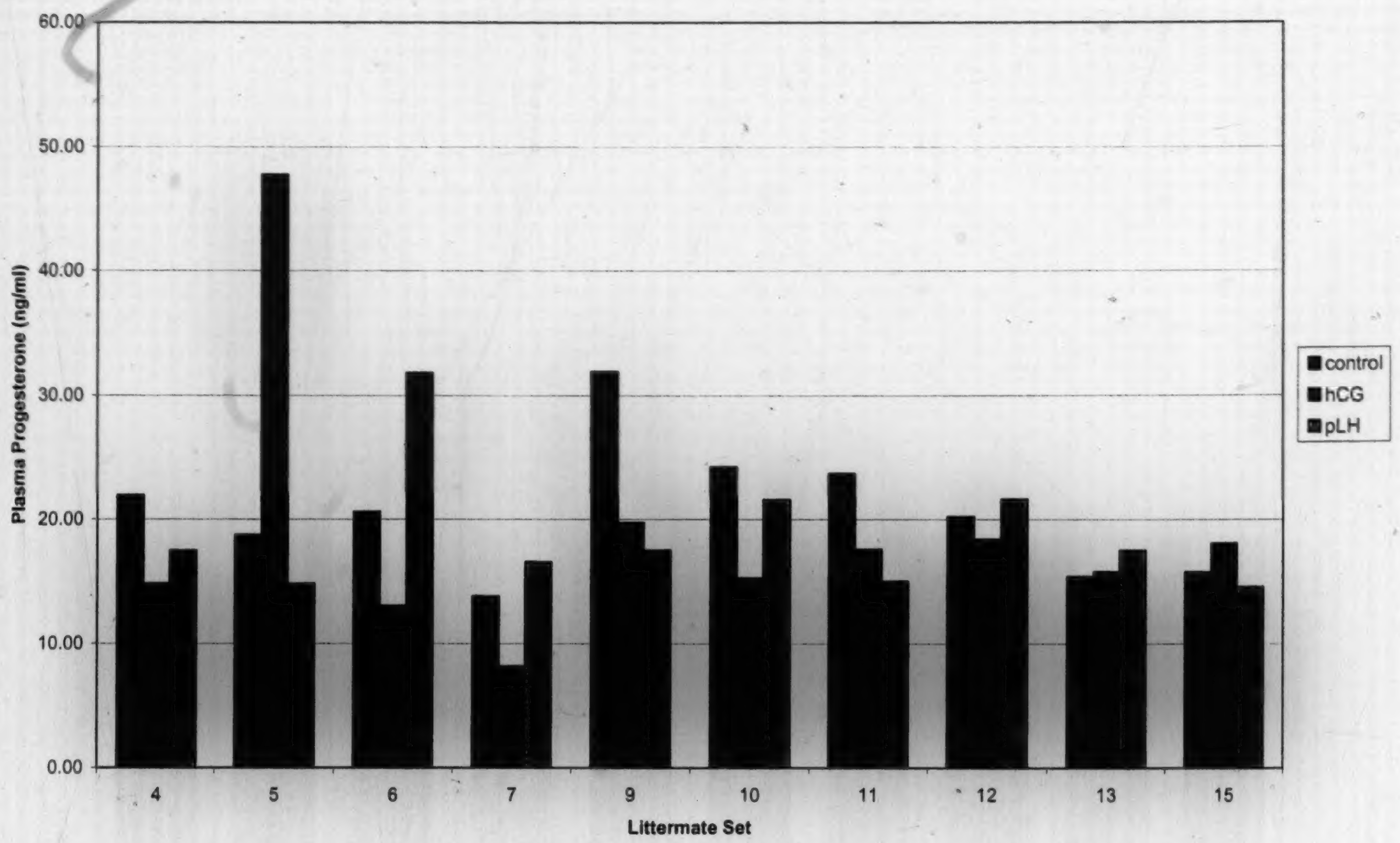

Figure 3.3: Plasma progesterone concentrations for each animal within a littermate set at day 5.9 (± 0.03) of gestation.

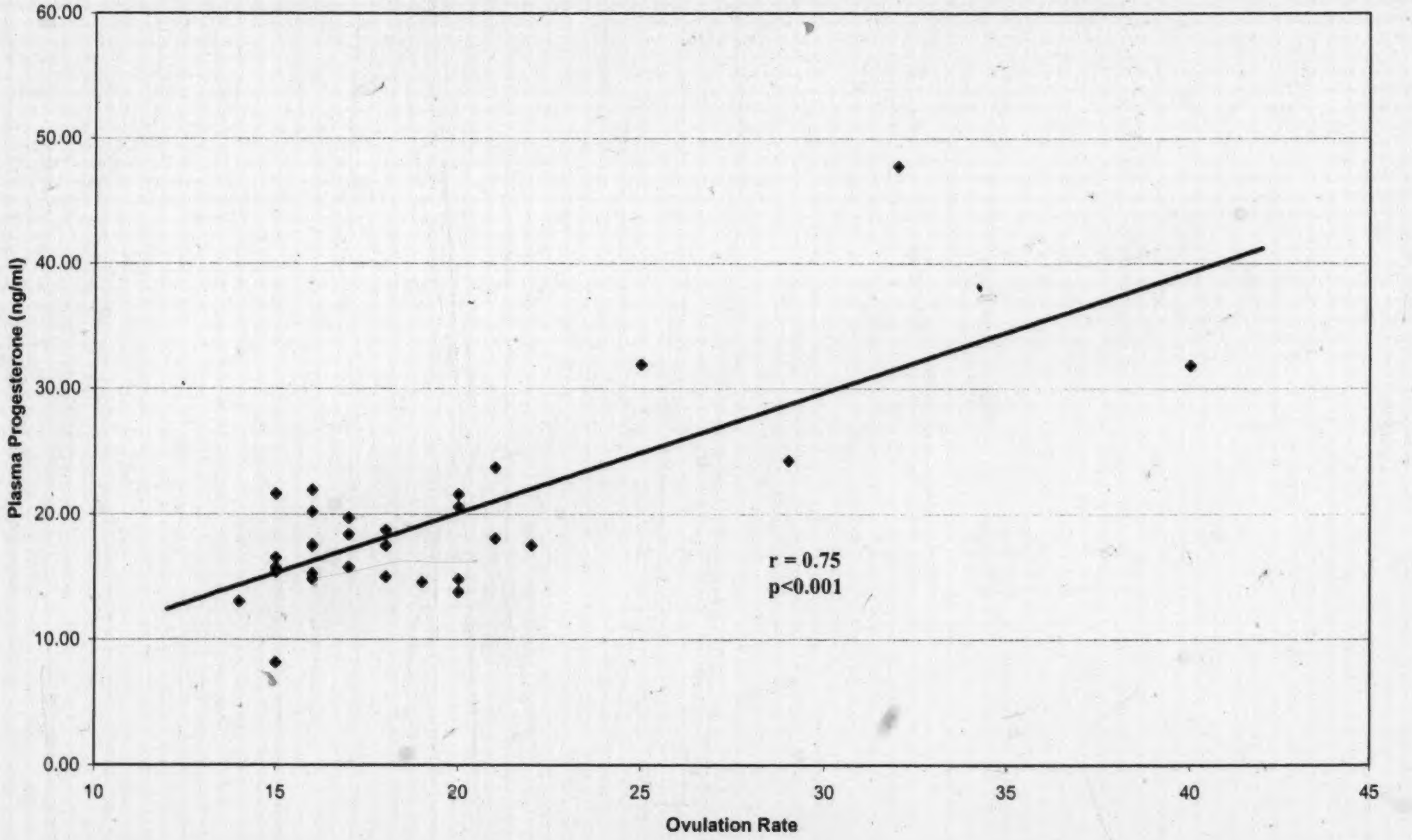

Figure 3.4: Relationship between ovulation rate and plasma progesterone concentration at day 5.9 (± 0.03) of gestation (n = 30).

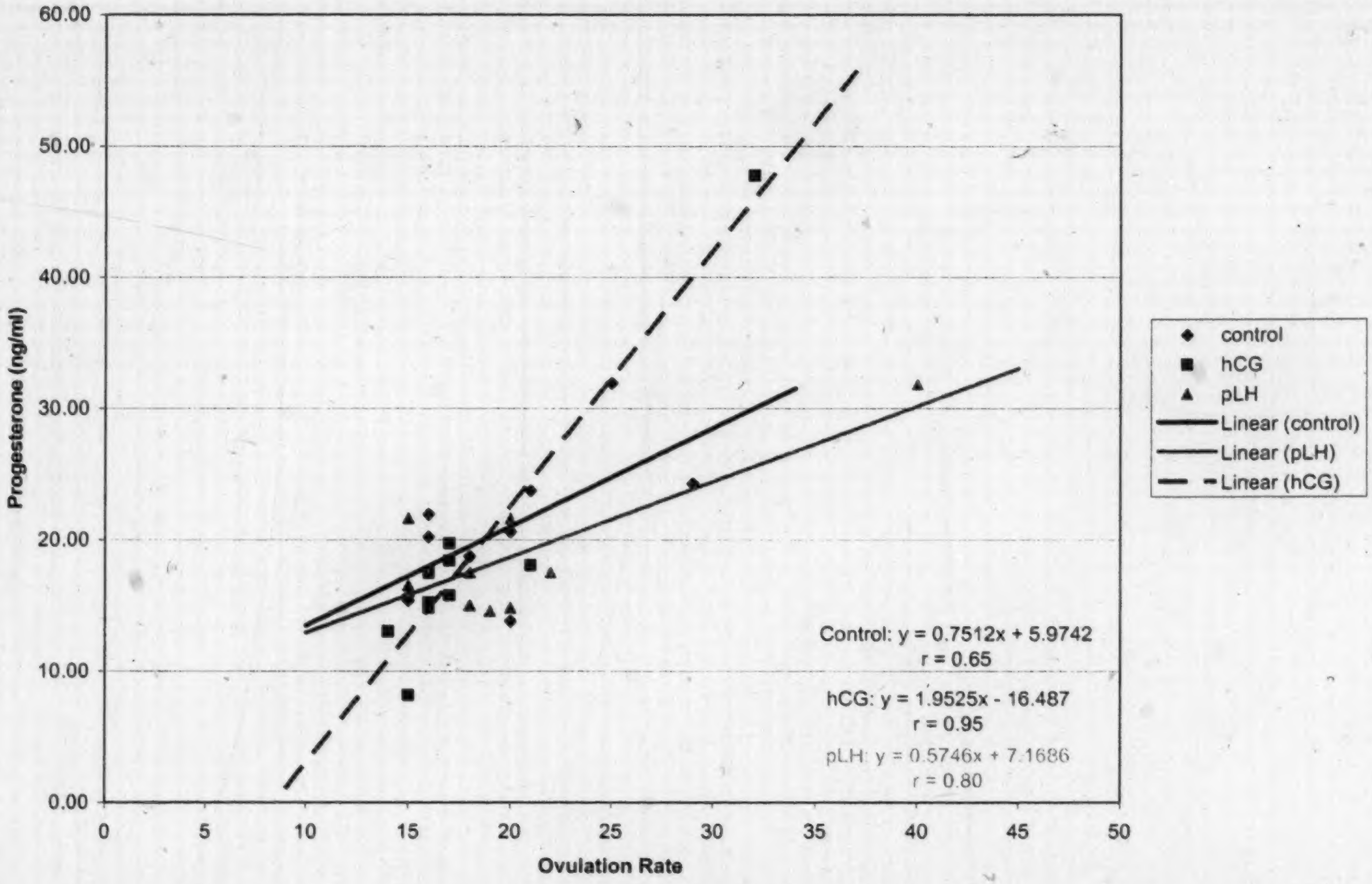

Figure 3.5: Relationship between ovulation rate and plasma progesterone concentration at day 5.9 (± 0.03) of gestation separated by treatment (n = 30).

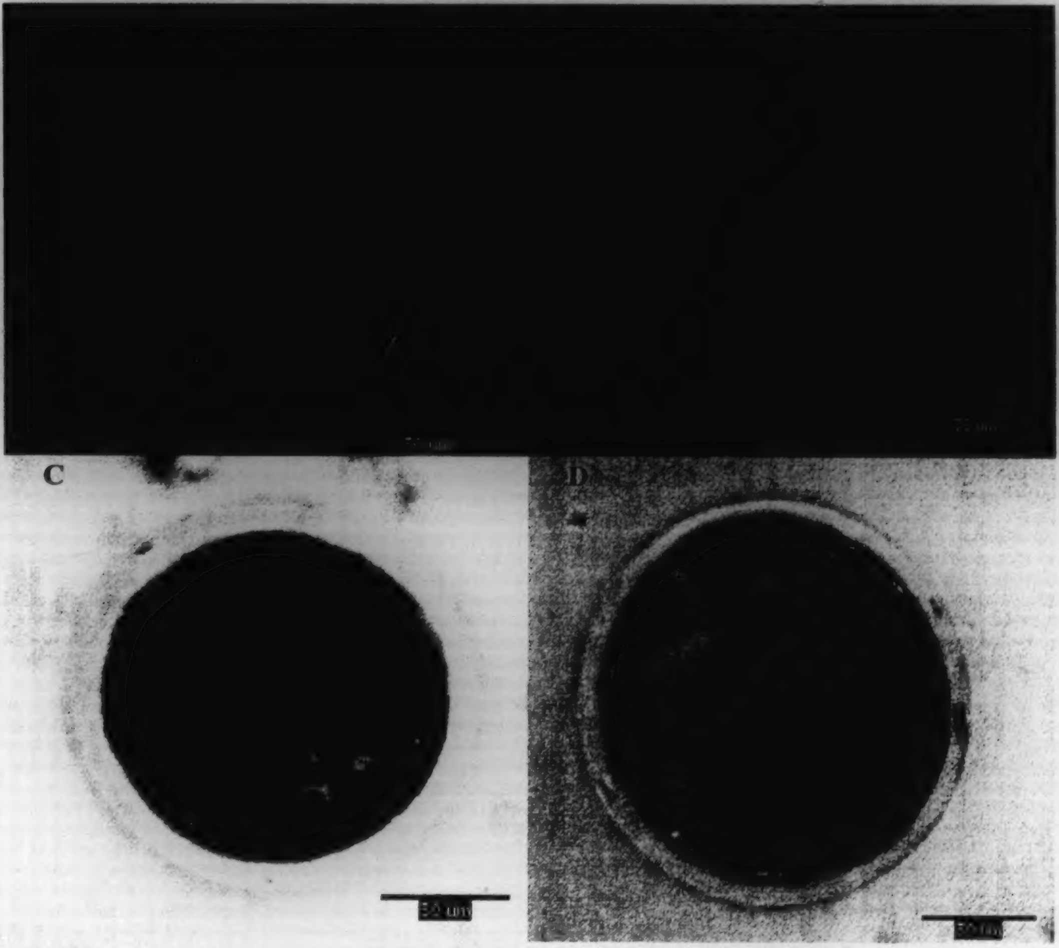

Figure 3.6: Representative pictures of early (A, B) and non-expanded blastocysts (C, D).

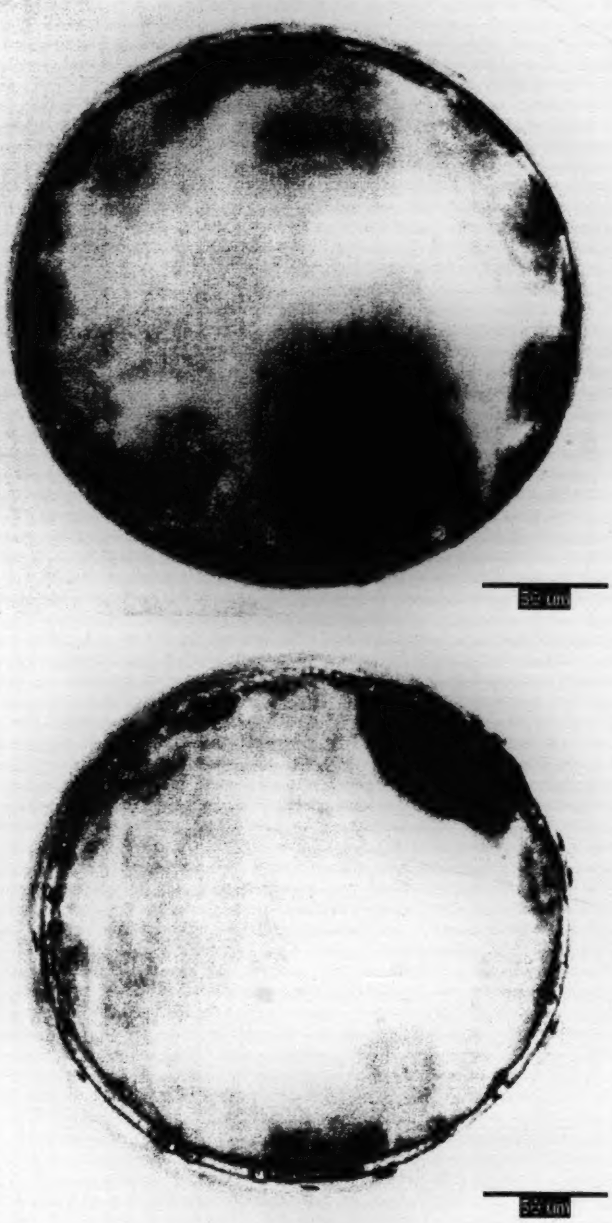

Figure 3.7: Representative pictures of expanded blastocysts.

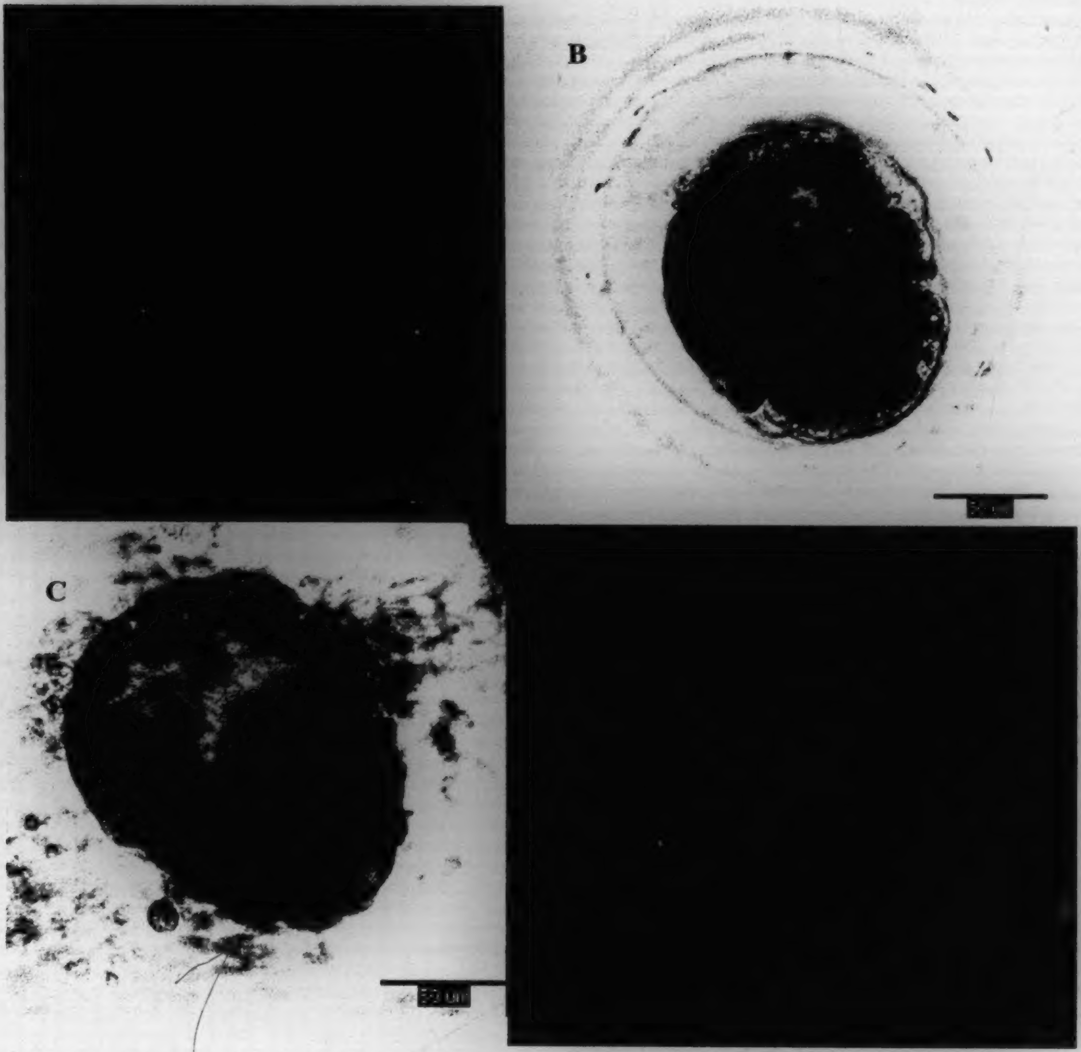

Figure 3.8: Representative pictures of de-expanded (A, B) and hatched blastocysts (C, D).

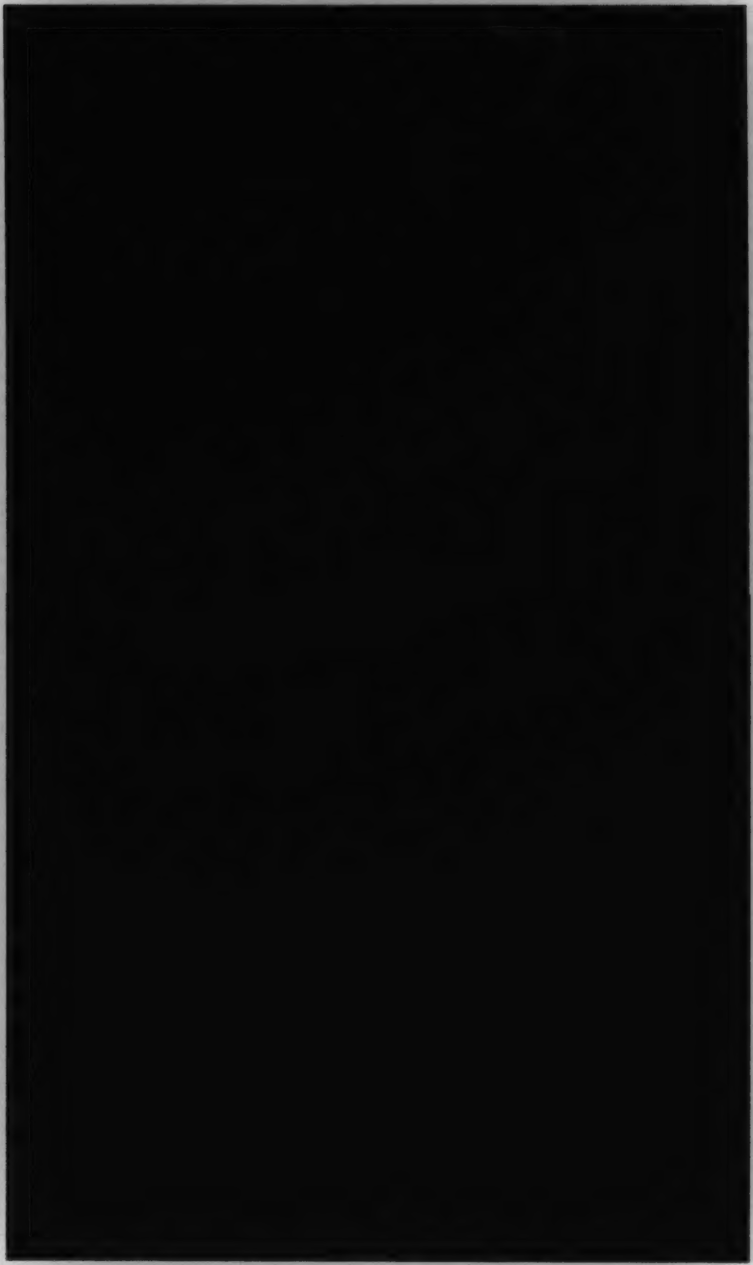

Figure 3.9: Examples of differentially stained expanded blastocysts. Cells appearing pink are stained with propidium iodide, cells appearing blue are stained with bisbenzimide, and cells appearing purple are stained with both.

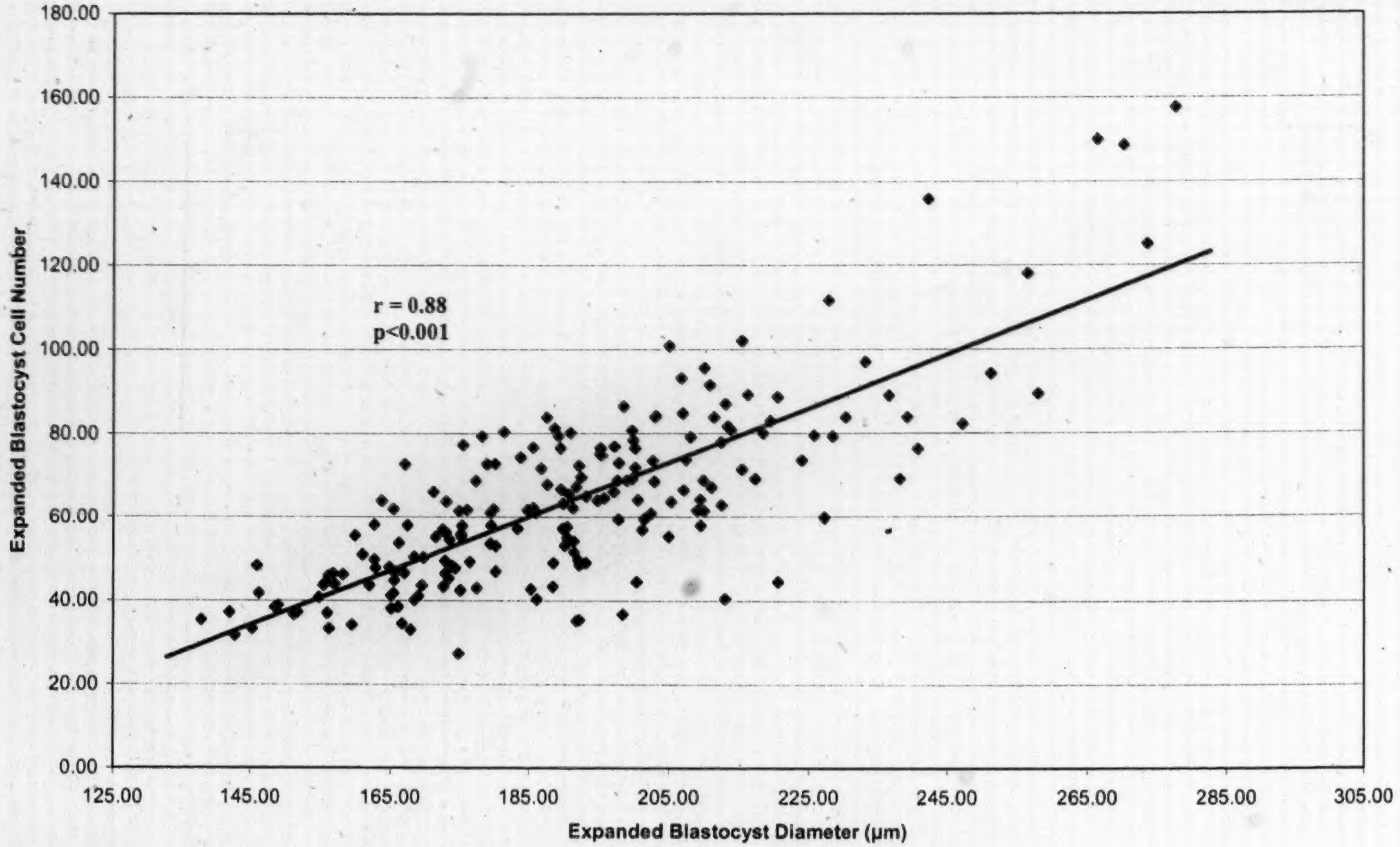

Figure 3.10: Relationship between individual expanded blastocyst diameter and total cell number.

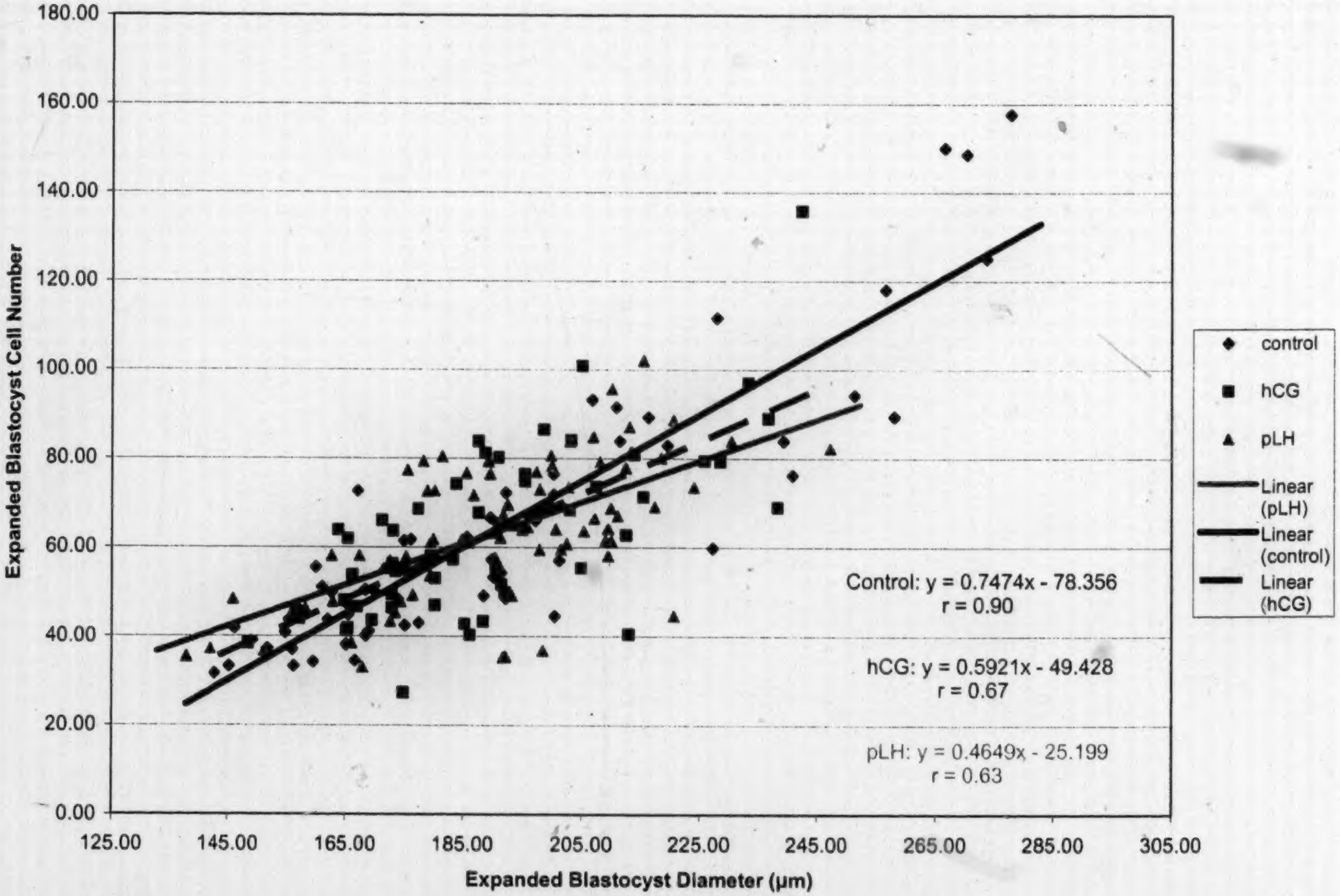

Figure 3.11: Relationship between individual expanded blastocyst diameter and total cell number separated by treatment.

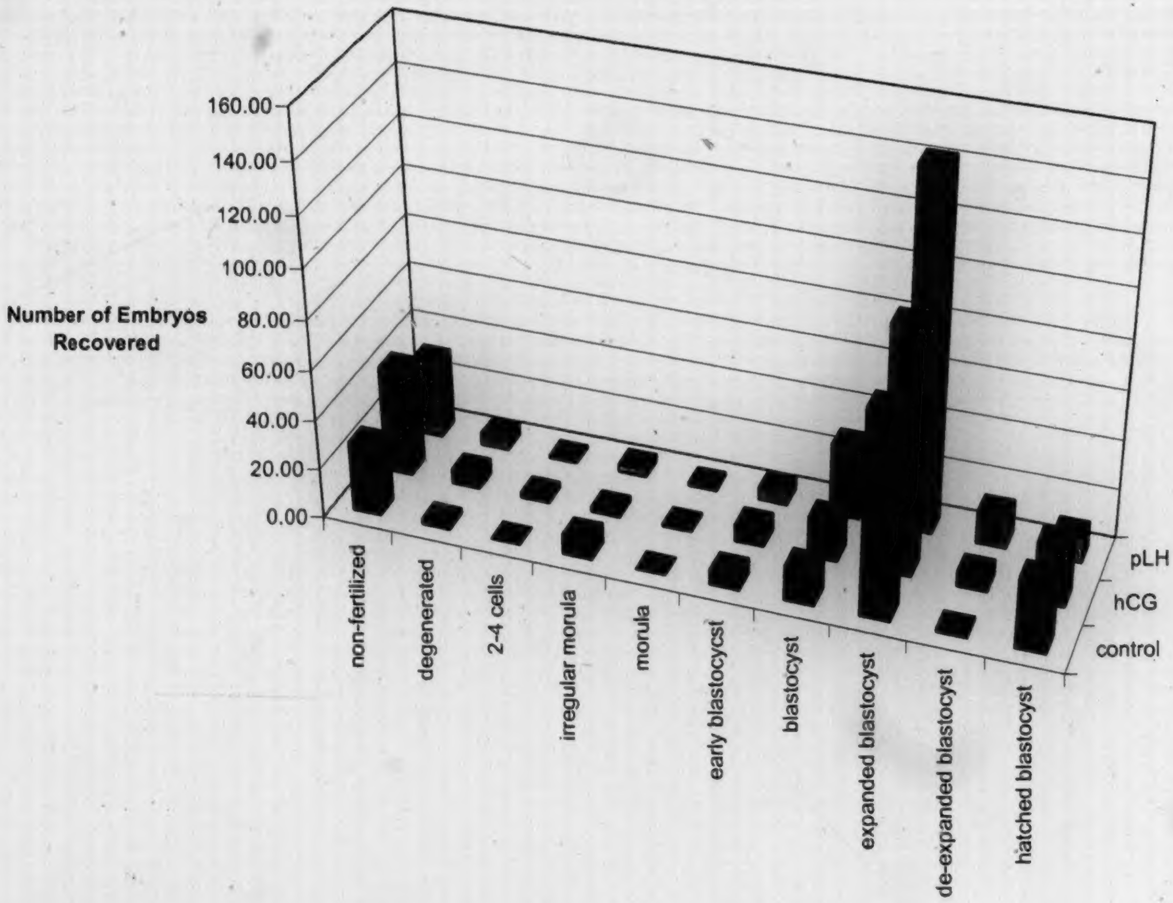

Figure 3.12: The stage of the embryos recovered for each treatment at day 5.9 (± 0.03) of gestation.

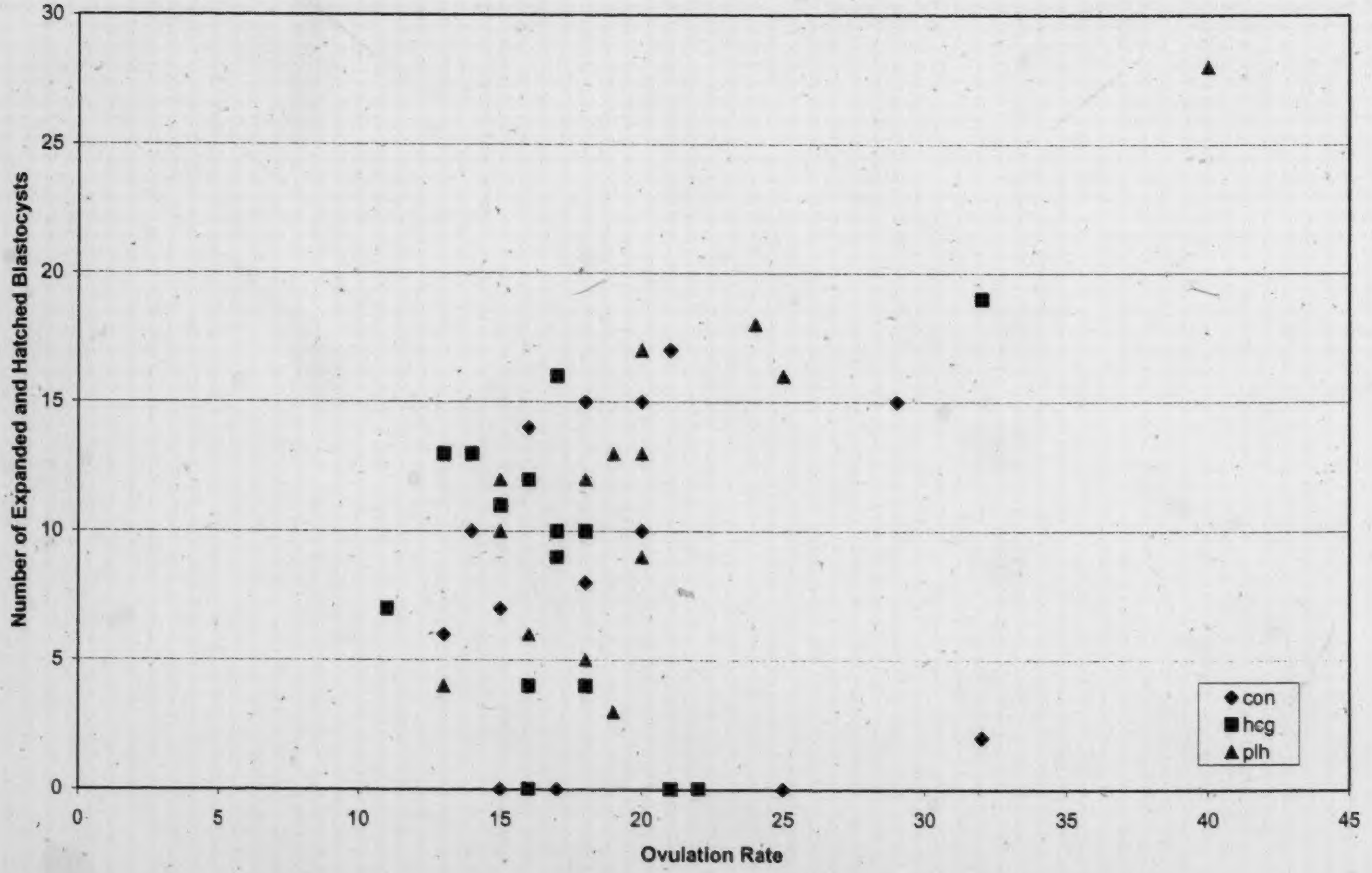

Figure 3.13: Relationship between the number of expanded and hatched blastocysts recovered at day 5.9 (± 0.03) of gestation, and ovulation rate.

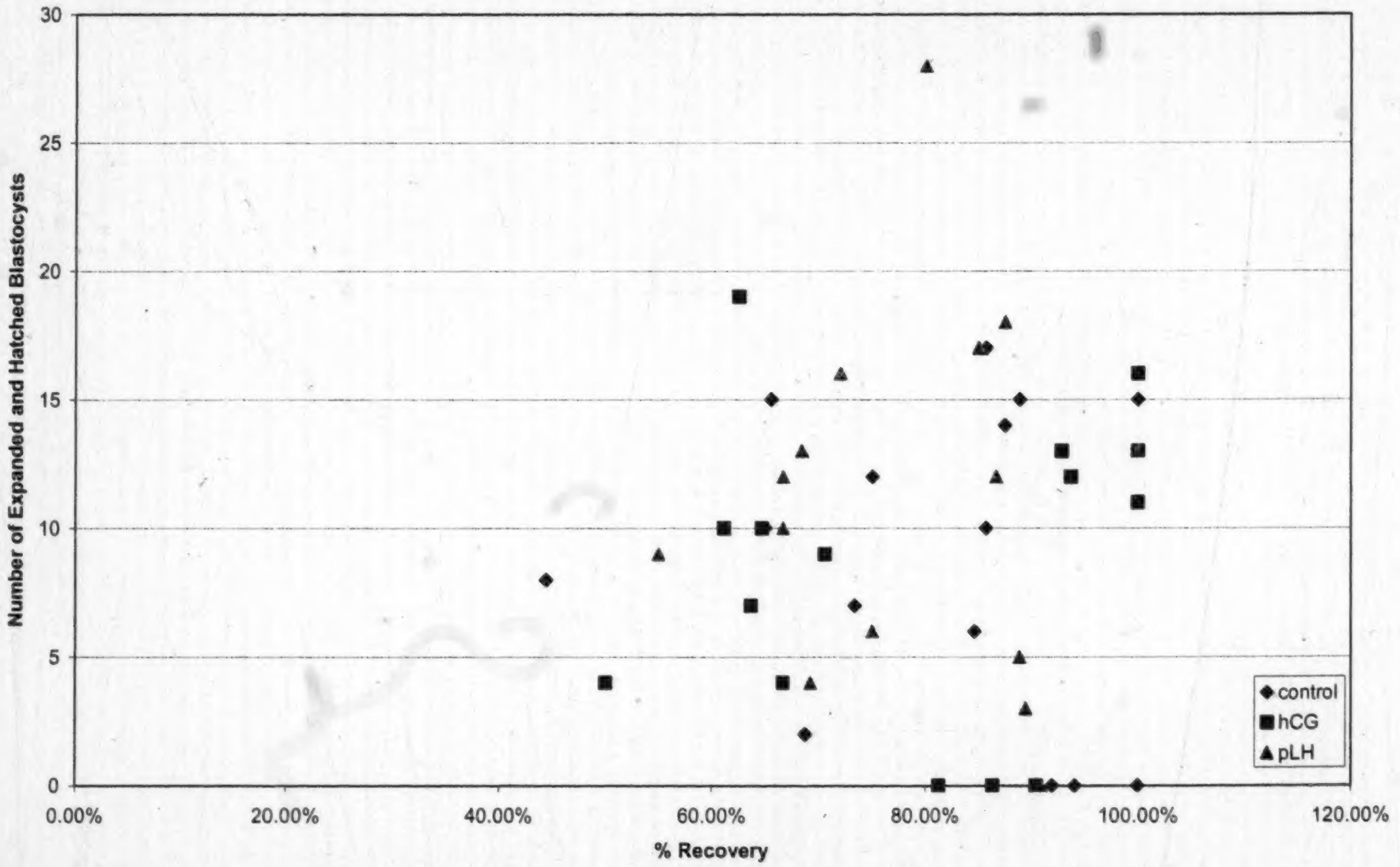

Figure 3.14: Relationship between the number of expanded and hatched blastocysts recovered at day 5.9 (± 0.03) of gestation, and the rate of recovery.

CHAPTER 4
GENERAL DISCUSSION AND CONCLUSIONS

4.1 General Discussion

High rates of reproductive efficiency are an essential component of successful swine production. To achieve this, producers must identify and incorporate breeding herd management programs that allow them to consistently achieve maximum conception rates, farrowing rates, and litter sizes and ensure optimal resource management. As part of this strategy, the use of artificial insemination (AI) has been increasing in the swine industry (Burke 1999). It enhances genetic improvement, and when coupled with methods to control the timing of estrus and ovulation, insemination can be performed at fixed times to reduce the amount of labour required for estrus detection and breeding (Huhn *et al.* 1996). As well, the emerging application of embryo transfer protocols in the swine industry for the introduction of new genetics into breeding herds in an efficient and biosecure manner, also benefits from the use of exogenous hormones to control the timing of ovulation (Hazeleger *et al.* 1994a; Hazeleger and Kemp 1994b). Ovarian control is not only necessary to obtain embryos at the appropriate stage of development to be transferred successfully, it also facilitates the synchronization of the embryo recipients' reproductive systems so that they can successfully nurture and maintain the embryos of interest until parturition to produce healthy, viable offspring.

As in any aspect of livestock production, there are various costs associated with the type of breeding protocol employed in a production system. If natural mating is used, costs include the labour to supervise breeding and maintaining a large population of boars on-site to accommodate the fact that a boar only breeds a single female at a time. With AI, fewer boars need to be kept on-site. However, there are labour and material costs associated with collecting and processing semen, or costs of purchasing semen, as well as expenses associated with AI supplies and employing well-trained staff who are able to perform the inseminations correctly. Moving to a fixed-time AI protocol, additional costs are incurred with the use of exogenous hormone treatment. Yet, with each technique, benefits in other areas aim to offset these costs. It was shown by Flowers and Alhusen

(1992) that in large breeding herds, costs of AI in terms of semen collection and processing were offset by the reduced labour demand during breeding. As presented at the 2007 Swine Breeding Management Workshop (Edmonton, Alberta), Donald Levis showed that net profits can be increased by decreasing the number of inseminations per animal, which justify the higher costs associated with fixed-time AI. Based on an economic analysis comparing a standard AI protocol to a fixed-time AI protocol in a breeding herd of 250 sows (Bioniche technical information), it is predicted that producers can save $0.88 per piglet for a sow operation, $1.32 per pig from weaned sow to feeder, and $2.23 per pig from farrow to finish. However, when deciding what strategy to use, a cost-benefit analysis must be done in any individual situation to determine the best protocol to use for that production system.

The main use of gilts in breeding herds is for replacement of culled breeding stock (Foxcroft *et al.* 2004). It is therefore a priority to be able to quickly and properly introduce these animals into the management flow of the breeding herd. As reviewed by Foxcroft *et al.* (2004), with proper management, a good population of cyclic gilts can be established but variability of the animals' estrous cycles will remain; although the majority of animals will respond to proper management to stimulate puberty, there is variation in the timing of this response (75% of gilts reach puberty within 40 days of boar exposure that started at day 140 of age (Foxcroft *et al.* 2004)). To meet breeding targets, one needs to be able to reliably predict the estrus of these cyclic gilts to properly introduce them into breeding groups within the herd. The results of the research presented in this thesis shows that pLH, when used in cyclic gilts, can induce a tight interval during which ovulation occurs. Therefore, it was determined that synchronization of gilts with pLH could offer reliability, as well as allow for fixed-time AI, without detrimentally affecting the quality of embryos produced and, therefore, the farrowing rate or litter size. As discussed, the use of pLH has higher costs associated with it, but in principle, utilizing pLH treatment has the potential to decrease the size of the gilt pool that would be needed to be maintained as replacements. The cost savings in labour, as well as selecting and maintaining fewer gilts, easily outweighs the higher product costs related to fixed-time AI.

In order to maximize the potential benefits of a fixed-time AI protocol, ideally only a single insemination should be necessary. Initial studies looking at pLH use in weaned sows found one insemination can be successful (Cassar *et al.* 2005). The present study incorporated double fixed-time inseminations, typical of procedures associated with embryo transfer protocols. This was done as the aim was to collect and evaluate embryonic quality at day 6 of gestation and we did not want the number of potential embryos to be compromised by limiting the animals to one insemination. Therefore, a single insemination protocol was not directly tested. All but one of the pLH-treated animals in the studies described in Chapters 2 and 3 received two inseminations at 32 and 40 hours following injection, resulting in the second insemination falling approximately 12 hours prior to or 4 hours following ovulation. In the animal that received a single insemination at 32 hours (ovulation was confirmed prior to the second scheduled insemination at 40 hours), the insemination occurred approximately 3 hours prior to ovulation. Ideal AI timing for gilts has been reported to be 12 to 0 hours prior to ovulation and is influenced by the age of semen (Waberski *et al.* 1994). Other studies indicate that this interval could be extended to 28 hours prior to and 4 hours after ovulation (Nissen *et al.* 1997). According to these results, for a pLH based fixed-time single insemination protocol utilizing good quality semen, the insemination needs to occur within the period used in this study. If for example, a single insemination was given at 36 hours following pLH treatment, all animals in this study would have been inseminated at 16 hours prior to or one hour following ovulation, which falls within the optimum insemination period with no animals being missed. Although one pLH-treated animal produced a high number of unfertilized oocytes, it appears that this was not due to the timing of the insemination, which was administered appropriately as determined by ultrasonography, but a characteristic of the gilt itself. It was determined that the number of viable embryos and expanded and hatched blastocysts recovered tended to be influenced by a littermate effect. No one factor is able to explain the reason for this difference except for litter of origin.

Although the timing of insemination used was appropriate for the majority of animals, there are confounding factors that influence the results, so ultimately there will undoubtedly be failure of fixed-time insemination in some animals no matter the treatment applied. Further to this, it was observed that the display of standing estrus for treated animals was quite variable. With this in mind, it is important to emphasize that for fixed-time AI it is the timing of treatment that determines when the insemination occurs and not onset of estrus. The pLH-treated animals in this study that failed to display normal estrus characteristics still ovulated within the interval of other treated animals and produced viable and well developed embryos. However, the effect of the boar leading up to ovulation and during inseminations has been well researched (see review by Langendijk *et al.* 2005), therefore, removing boar exposure all together should be researched further.

Treatment with altrenogest, eCG, and pLH followed by two inseminations produced litters primarily made up of viable expanded blastocysts, which are ideal for embryo transfer procedures. The pLH also provided a benefit over hCG by more consistently stimulating ovulation in a predictable manner, and tending to result in more viable embryos. The limited variation in timing of ovulation following pLH treatment could potentially improve the synchrony between donor and recipients in embryo transfer protocols, providing a better environment for transferred embryos. In addition, hCG is not approved for use in swine in Canada, whereas pLH is. Therefore, the use of pLH in protocols for fixed-time AI and embryo transfer can make these technologies more accessible to industry. However, depending on the application of pLH, it is possible to limit the treatments applied. For example, in a production system looking to implement a fixed-time AI protocol, pLH could be used on its' own if adequate estrus detection records are kept, and the technician is aware of what stage of the estrous cycle the animal is in. If the point in the estrous cycle is unknown, pLH should be combined with other synchronization techniques (i.e. altrenogest) to ensure the pLH treatment is effective.

A dose of 5 mg of pLH was used in this study, based on the reliability of this dosage in previous research (Cassar *et al.* 2005; Candini *et al.* 2001; Viana *et al.* 2002), the recommendation by the pLH manufacturer that 5 mg be used, and the response measured in the pilot study. Conversely, potency studies have seen relatively consistent results using 2.5 mg (Viana *et al.* 2005; Bennett-Steward *et al.* 2007). Reducing the dosage may be one way of reducing cost associated with treatment. For example, using a 2.5 mg pLH dosage results in a predicted savings of $1.02 per piglet for the sow operation, $1.46 per pig from weaned sow to feeder, and $2.37 per pig feeder to finish, based on a 250 sow breeding herd using a one insemination fixed-time AI protocol (Bioniche technical information). However, no research with a reduced dosage has been done in cyclic gilts, and the effectiveness of this lower dosage would need to be investigated further.

It is interesting to note the increasing use of porcine follicle stimulating hormone (pFSH) in hormonal stimulation protocols in cattle (Staigmiller *et al.* 1995; Goulding *et al.* 1991), while use of this product in swine is limited. This is unusual considering that pFSH has shown promising results in cattle when compared to eCG (Staigmiller *et al.* 1995; Goulding *et al.* 1991), and there are reports of eCG detrimentally affecting embryo quality in pigs (Hazeleger *et al.* 2000). As treatment with pLH tended to result in more viable embryos when compared to hCG in this study, a similar or even additive result could be obtained with the use of pFSH (an endogenous porcine hormone) compared to eCG. Another possible advantage of using pFSH is that it might offer an alternative to eCG treatment which has been shown to produce high variability in individual response to the hormonal treatment, namely in ovulation rate (Fujino *et al.* 1996). From the limited studies conducted in swine involving pFSH, it was noted to have the ability to stimulate follicular growth in prepubertal gilts (Guthrie *et al.* 1990) and can stimulate superior follicular growth overall compared to eCG (Bolamba *et al.* 1996; Guthrie *et al.* 1997). However, these results are not ideal, as it was observed that pFSH stimulated growth of medium, but not large follicles (Guthrie *et al.* 1990). Moreover, although stimulation of follicular growth was observed, many of these follicles failed to ovulate after hCG treatment (Guthrie *et al.* 1997). As well, pFSH treatment is given in multiple injections rather than a single dose like eCG (Guthrie *et al.* 1990, 1997). As Guthrie *et al.* (1990) state, additional research is needed to fully understand if and how exogenous pFSH can be used in pigs. However, based on the substantial results obtained in cattle, the use of pFSH in combination with pLH may offer an alternative hormonal stimulation protocol that is more ideal than the use of eCG and hCG.

A notable response observed in the pLH-treated animals was that they consistently ovulated smaller follicles. It has been shown that the height of the LH surge is positively correlated with insulin-like growth hormone I (IGF-I), which in turn can influence follicular size (Madej *et al.* 2005). Therefore, it could be that the greater LH concentrations observed in pLH-treated animals could positively interact with IGF-I to increase follicular size. However, because pLH-treated animals also ovulated earlier, IGF-I may not have had enough time to influence follicular growth. Furthermore, delaying breeding in sows following lactation resulted in larger follicles and increased litter size (Foxcroft *et al.* 2007). So it is both surprising and encouraging to note in this study that the smaller follicular diameter did not influence embryo quality, and pLH-treated animals produced as many viable embryos as control animals that were seen to have a larger average follicular size prior to ovulation.

Embryo quality at day 6 of development as determined by morphological evaluation (total cell counts and embryo diameter), was not affected by hormonal treatment in this study. To confirm this finding, the timing of embryo collection and evaluation should be extended (for example, day 30 of gestation, or full term) to ensure that there is indeed no effect of hormonal treatment on embryo quality, as there may be embryonic loss between day 5 and day 30 of gestation (Geisert and Schmitt 2002). Cell number and blastocyst expansion have often been used as indicators of embryo quality (Machàty *et al.* 1998; Kim *et al.* 2005; Ock *et al.* 2007; Hazeleger *et al.* 2000; Fujino *et al.* 2006); however, any morphological evaluation can be subjective, particularly when these measurements are performed manually. The subjectiveness of these approaches can be reduced by using one person to perform all measurements which is not always practical, or by using computer software with standardized programs to obtain these measurements, as was done in this study. Still, it would be ideal to be able to associate these measures with other parameters that indicate embryo quality. One suggestion for the continuation of this research would be to obtain diameter measurements and then perform embryo transfer so that the rate of development of these embryos to day 30 of gestation or to term could be determined and correlated with this parameter.

The use of the ratio between the inner cell mass (ICM), trophectoderm (TE), and total cell numbers has been established as an important parameter in evaluating bovine embryos, as a skewed ratio in the number of TE cells to total cells has been associated with Large Offspring Syndrome (Russell *et al.* 2006). In swine, the size of the ICM in the prolific Meishan breed is similar to other breeds but the number of cells in the trophectoderm TE is less (Rivera *et al.* 1996). The number of ICM cells appears to be an important factor for blastocyst viability, and significant loss of ICM cells can lead to developmental delay and failure of the embryo to implant (Tam 1988). In light of this, establishing a reliable method for differential staining in porcine embryos would be advantageous for defining early porcine embryo quality. As a part of this study, a portion of the embryos collected were frozen to hopefully allow for molecular analysis in the future. Such genetic analysis has been performed for day 8 bovine blastocysts using RT-PCR and quantitative PCR, looking at expression of developmentally important genes (Russell *et al.* 2006). However, as discussed in Chapter 1, indicators of embryo quality in terms of gene expression are limited for the early porcine embryo. Analysis of these embryos using techniques such as microarray may highlight possible genetic markers of embryo quality. These markers may then be used in combination with morphological measures such as total cell counts, cell ratios, and blastocyst expansion to provide a more comprehensive understanding of embryo quality.

4.2 Conclusions

The research described establishes the basis for pLH use in cyclic gilts. Reliable and consistent results were obtained for the treatment-ovulation interval, indicating that this product could be used in fixed-time AI protocols applying only a single insemination. As well, pLH treatment did not detrimentally influence embryo quality, and provided more optimal results than hCG. Therefore, the potential use of pLH in embryo transfer procedures is likely superior to hCG, and should be investigated further. Large-scale production studies should be conducted to confirm that this product is appropriate for use in a commercial production setting. As well, studies are required to evaluate embryonic survival at later stages of gestation following pLH treatment to establish if the parameters used at day 6 to evaluate embryo quality are truly indicative of the viability of those embryos. Molecular analysis of early embryos will also provide insight into the reliability of morphological evaluation of embryos in early stages of development.

4.3 References

Bennett-Steward, K., Cassar, G., Plante, C., Friendship, R.M., Zak, L. 2007. Ovulation induction protocol using equine chorionic gonadotropin and porcine luteinizing hormone in weaned sows. *J. Swine Health Prod.* 15: 194-197.

Bioniche technical information: Financial Model. Available at URL: http://www.singletimedbreeding.com/pdfs/Save_with_SingleTimedBreeding.xls

Bolamba, D., Dubuc, A., Dufour, J. J., Sirard, M- A. 1996. Effects of gonadotropin treatment on ovarian follicle growth, oocyte quality, and in vitro fertilization of oocytes in prepubertal gilts. *Theriogenology.* 46:717-726.

Burke, P. 1999. The successful introduction of AI. *Advances in Pork Production.* 10:53-62.

Candini, P.H., Moretti, A.S., Silveria P.R.S., Zanella, E., Viana, C.H.C., Santos, I.C.C. 2001. Utilization of a protocol of synchronization of ovulation, induced by porcine LH, to perform insemination programs in fixed time, with one or two inseminations per sow. Sixth International Conference on Pig Reproduction, Columbia MO. pp. 99.

Cassar, G., Kirkwood, R.N., Bennett-Steward, K., Friendship, R.M. 2005. Towards timed single insemination in sows. Proceedings of the 36th Annual Meeting of the American Association of Swine Veterinarians, Toronto, ON. pp. 353-356.

Flowers, W.L., Alhusen, H-D. 1992. Reproductive performance and estimates of labor requirements associated with combinations of artificial insemination and natural service in swine. *J. Anim. Sci.* 70:615-21.

Foxcroft G., Patterson, J., Beltranena, E., Pettitt, M. 2004. Identifying the true value of effective replacement gilt. Proceedings of the Manitoba Swine Seminar, Winnipeg, MB.

Foxcroft, G.R., Vinsky, M.D., Paradis, F., Tse, W-Y., Town, S.C., Putman, C.T., Dyck, M.K., Dixon, W.T. 2007. Macro-environment effects on oocytes and embryos in swine. *Theriogenology.* Epub.

Fujino, Y., Nakamura, Y., Kobayashi, H., Kikuchi, K. 2006. Relationship between time elapsed after human chorionic gonadotropin administration and developmental stage in porcine embryos collected from prepubertal gilts. *J. Reprod. Dev.* 52:267-275.

Geisert, R.D., Schmitt, R.A.M. 2002. Early embryonic survival in the pig: Can it be improved? *J. Anim. Sci.* (E. Supplement 1) 80:E54-E65.

Goulding, D., Williams, D.H., Roche, J.F., Boland, M.P. 1991. Superovulation in heifers using either pregnant mare's serum gonadotrophin or follicle stimulating hormone during the mid luteal stage of the estrous cycle. *Theriogenology*. 36:949-958.

Guthrie, H.D., Bolt, D.J., Cooper, B.S. 1990. Effects of gonadotropin treatment on ovarian follicle growth and granulosal cell aromatase activity in prepubertal gilts. *J. Anim. Sci.* 68:3719-3726.

Guthrie, H.D., Pursel, V.G., Wall, R.J. 1997. Porcine follicle-stimulating hormone treatment of gilts during an altrenogest-synchronized follicular phase: Effects on follicle growth, hormone secretion, ovulation, and fertilization. *J. Anim. Sci.* 75:3246-3254.

Hazeleger, W., Bouwman, E.G., Noordhuizen, J.P.T.M., Kemp, B. 2000. Effect of superovulation induction on embryonic development on day 5 and subsequent development and survival after nonsurgical embryo transfer in pigs. *Theriogenology*. 53:1063-1070.

Hazeleger, W., Kemp, B., Soede, N.M., Noordhuizen, J.P.T.M., van der Lende, T. 1994a. Transcervical embryo collection and reproductive performance of sows with resectioned uteri. *Reprod. Dom. Anim.* 29:38-45.

Hazeleger, W., Kemp, B. 1994b. Farrowing rate and litter size after transcervical embryo transfer in sows. *Reprod. Dom. Anim.* 29:481-487.

Huhn, U., Jochle, W., Brussow, K.P. 1996. Techniques developed for the control of estrus, ovulation and parturition in the East German pig industry: A review. *Theriogenology*. 46:911-24.

Kim, H-s., Lee, G-s., Hyun, S-h., Nam, D-h., Lee, S-h., Jeong, Y-W., Kim, S., Kim, J-h., Kang, S-k., Lee, B-c., Hwang, W-s. 2005. Embryotropic effect of glycosaminoglycans and receptors in development of porcine pre-implantation embryos. *Theriogenology*. 63:1167-1180.

Langendijk, P., Soede, N.M., Kemp, B. 2005. Uterine activity, sperm transport, and the role of boar stimuli around insemination in sows. *Theriogenology*. 63:500-513.

Levis, D.G. 2007. AI timing, fertility and AI doses. Presented at the Swine Breeding Management Workshop, Edmonton, AB.

Machàty, Z., Day, B.N., Prather, R.S. 1998. Development of early porcine embryos in vitro and in vivo. *Biol. Reprod.* 59:451-55.

Madej, A., Lang, A., Brandt, Y., Kindahl, H., Madsen, M.T., Einarsson, S. 2005. Factors regulating ovarian function in pigs. *Domest. Anim. Endocrinol.* 29:347-61.

Nissen, A.K., Soede, N.M., Hyttel, P., Schmidt, M., D'Hoore, L. 1997. The influence of time of insemination relative to time of ovulation on farrowing frequency and litter size in sows, as investigated by ultrasonography. *Theriogenology.* 47:1571-82.

Ock, S-A., Lee, S-L., Kim, J-G., Kumar, B-M., Balasubramanian, S., Choe, S-Y., Rho, G-J. 2007. Development and quality of porcine embryos in different culture system and embryo-producing methods. *Zygote.* 15:1-8.

Rivera, R.M., Youngs, C.R., Ford, S.P. 1996. A comparison of the number of inner cell mass and trophectoderm cells of preimplantation Meishan and Yorkshire pig embryos at similar developmental stages. *J. Reprod. Fertil.* 106:111-116.

Russell, D.F., Baqir, S., Bordignon, J., Betts, D.H. 2006. The impact of oocyte maturation media on early bovine embryonic development. *Mol. Reprod. Dev.* 73:1255-1270.

Staigmiller, R.B., MacNeil, M.D., Bellows, R.A., Short, R.E., Phelps, D.A. 1995. The effect of estrus synchronization scheme, injection protocol and large ovarian follicle in response to superovulation in beef heifers. *Theriogenology.* 43:823-834.

Tam, P.P.L. 1988. Postimplantation development of mitomycin c-treated mouse blastocysts. *Teratology.* 37:205-212

Viana, C.H.C., Candini, P.H., Gama, R.D., Carbone, A., Santos, I.C.C., Vianna, W.L., Barnabe, R.C. 2002. Evaluation of a synchronization of ovulation protocol in cyclic sows. Proceedings of the International Pig Veterinary Congress, Ames, Iowa #678.

Viana, C.H.C., Castricini, E.S.C., Santos, I.C.C. 2005. Determination of the minimum porcine LH dose on synchronization of ovulation in sows. Presented at the International Congress on Pig Production, Amsterdam.

Waberski, D., Weitze, K.F., Lietmann, C., Lubbert zur Lage, W., Bortolozzo, F.P., Willmen, T., Petzoldt, R. 1994. The initial fertilizing capacity of long term-stored liquid boar semen following pre- and postovulatory insemination. *Theriogenology.* 41:1367-77.